University of Alberta

The Interaction of Benzodiazepines with the $GABA_A$ Receptor of Mammalian Brain

by

Lori Lee Duncalfe ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

Edmonton, Alberta

Spring 1996

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ISBN 0-612-10582-2

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Degree: Doctor of Philosophy

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ABSTRACT

The α-subunit of the γ-aminobutyric acid A ($GABA_A$) receptor has been photoaffinity labelled by [^{3}H]flunitrazepam. To identify the specific site of photoincorporation, labelled $GABA_A$ receptors from bovine cerebral cortex were subjected to cyanogen bromide cleavage and the resulting peptides were purified by immunoprecipitation with an anti-flunitrazepam polyclonal serum. A major 5.4 kDa photolabelled glycosylated peptide was resolved by reverse phase HPLC of the immunopurified peptides and subjected to direct amino acid sequencing. Based on the mass of peptides that can be generated from the α subunits and potential glycosylation sites, the pattern of release of radioactivity during Edman degradation of the photolabelled peptide was mapped to the known sequence of the receptor subunit. The major site of photoincorporation by [^{3}H]flunitrazepam is shown to be α subunit His102.

Cortical and cerebellar bovine $GABA_A$ receptors, photoaffinity labelled by an agonist ([^{3}H]flunitrazepam) or by a partial inverse agonist ([^{3}H]Ro15-4513), were subjected to specific chemical cleavage using hydroxylamine and the resulting peptides were mapped to the amino acid sequences of $GABA_A$ receptor subunits. It is shown that photoaffinity labelling sites for these benzodiazepines are localized to distinct domains of the α_1 subunit and that [^{3}H]Ro15-4513 photoaffinity labels a site on the α_6 subunit that is unique from its labelling site on the α_1 subunit.

The effects of sulfhydryl modification on benzodiazepine binding to $GABA_A$ receptors of bovine cerebral cortex have been studied. Dithiothreitol reduction inhibited the binding of [^{3}H]Ro15-4513 by both reducing affinity and binding site density. This

inhibition was prevented by pre-incubation with Ro15-4513 or flunitrazepam. The binding of [^{3}H]flunitrazepam was relatively insensitive to dithiothreitol. Alkylation of brain membranes by N-ethylmaleimide inhibited the binding of both radioligands with a similar concentration dependence, but via distinct mechanisms. Sulfhydryl modification of $GABA_A$ receptors is shown to have different effects on the binding of a benzodiazepine agonist and partial inverse agonist, suggesting differences in the modes of binding for the two ligands.

ACKNOWLEDGEMENTS

To Susan Dunn for your scientific expertise and the ability to formulate interesting and important, albeit challenging, pursuits.

To Ian Martin, Lawrence Smillie, Alan Bateson and Richard Olsen, for your keen interest, unique perspectives and valuable contributions to my research.

To the many individuals that I had the pleasure to work with over the years, for their valued input to my research and for providing such a friendly work environment.

To the faculty, staff and fellow students that are too numerous to list, for their support and kindness throughout my undergraduate and graduate program.

To my parents, Vernon and Jean, and to all my family and friends, for giving much encouragement throughout the many years of my schooling.

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LIST OF ABBREVIATIONS AND SYMBOLS

B_{max}	density of binding sites
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[-(cholimidopropyl)dimethyl ammonio]-1-propanesulfonate
CNBr	cyanogen bromide
DEP	diethylpyrocarbonate
DHP	α-dihydropicrotoxinin
DMCM	methyl 6,7-dimethoxy-4-ethyl-ß-carboline-3-carboxylate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
GABA	γ-aminobutyric acid
GTP	guanosine-5'-triphosphate
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine; serotonin
K_D	dissociation constant
MES	2-[N-morpholino]-ethanesulfonic acid
NMR	nuclear magnetic resonance
mRNA	messenger ribonucleic acid
N-EM	N-ethylmaleimide
N-Glycanase	peptide-N^4-(N-acetyl-ß-glucosaminyl)asparagine amidase
PITC	phenylisothiocyanate
PMSF	phenylmethylsulfonlyl fluoride

PTH	phenylthiohydantoin
Ro15-4513	ethyl-8-azido-5-6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5a][1,4]-benzodiazepine-3-carobxylate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBPS	tert-butylbicyclophosphorothionate
Tricine	*N*-tris(hydroxy-methyl)methyl glycine
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1

Introduction

GENERAL OVERVIEW

The benzodiazepines have been widely prescribed since their introduction in the 1960's and have remained some of the most useful psychoactive drugs due to their widespread application in clinical disorders. Our current understanding of the physiological mechanisms that underlie the actions of γ-aminobutyric acid (GABA) and the benzodiazepines has come from interdisciplinary investigations that originally began independently of one another. However, the disciplines converged in 1978, when it was demonstrated that the benzodiazepines elicited their effects by a specific interaction with GABA receptor proteins (Martin and Candy, 1978; Tallman et al., 1978; Briley and Langer, 1978). The neutral amino acid, GABA, is the major inhibitory neurotransmitter in the mammalian central nervous system and it elicits its effects via two distinct types of receptors: type A and B. The benzodiazepines interact with $GABA_A$ receptors, which are hetero-oligomeric proteins that form an integral chloride ion-selective channel. The $GABA_B$ receptors are coupled to certain calcium or potassium ion channels via GTP binding proteins and/or other messengers. Despite the vast amount of experimental work during the last few decades that has begun to elucidate the interaction between GABA and benzodiazepines, the molecular mechanisms underlying their interaction with the $GABA_A$ receptor are still unclear. The location of the benzodiazepine binding site(s) within the receptor complex is/are unknown and the mechanisms by which different types of benzodiazepines produce different pharmacological effects are poorly understood. This research has been directed towards resolving some of these fundamental properties,

with specific aims (1) to localize the benzodiazepine binding site(s) within the multi-subunit receptor complex by photoaffinity labelling with the photoactive derivatives, [^{3}H]flunitrazepam and [^{3}H]Ro15-4513, and (2) to identify structural determinants involved in benzodiazepine binding using protein modification techniques.

THE DISCOVERY OF GABA AS A NEUROTRANSMITTER

The presence of GABA in the mammalian brain was first demonstrated by two-dimensional paper chromatography of mouse brain extracts (Awapara et al., 1950) and glutamic acid was established as its precursor by an isotope derivative method (Roberts and Frankel, 1950; Udenfriend, 1950). The first implication that GABA played an inhibitory role in the mammalian central nervous system came from experiments that showed topically applied GABA to have an inhibitory effect on the electrical activity of the brain (Hayashi and Nagai, 1956). However, stronger evidence for an inhibitory effect of GABA was obtained with the demonstration that GABA was the main constituent of a brain extract, termed Factor I (Bazemore et al., 1957) that had previously been shown to exert an inhibitory influence on the firing of the crayfish stretch-receptor neuron (Florey, 1954). Using the crustacean model, invertebrate neurophysiologists further substantiated GABA as an inhibitory transmitter, by showing that the post-synaptic effects evoked by selective stimulation of inhibitory axons could be closely mimicked by exogenous GABA (Boistel and Fatt, 1958, Kuffler and Edwards, 1958, Takeuchi and Takeuchi, 1965). Subsequently, large quantities of GABA were

shown to be present in the crayfish inhibitory, but not excitatory, axons (Kravitz, 1963) and it was demonstrated that the release of GABA during inhibitory activity was calcium-dependent (Otsuka et al., 1966). Although early studies with mammalian models demonstrated that iontophoretically applied GABA had a powerful inhibitory effect on spinal neurons, the effect was dismissed as a non-specific depressant action that was unrelated to the mechanisms of neuronal inhibition (Curtis et al., 1959). However, the observation that a similar reversal potential characterized the voltage responses evoked by iontophoretically applied GABA and by inhibitory synaptic activity in cat cortical neurons led to the first suggestion that GABA was an important inhibitory transmitter in the mammalian brain (Krnjevic and Schwartz, 1967, Dreifuss et al., 1969).

Initially, the synaptic actions of GABA were thought to be mediated by a single class of receptors, coupled to ion channels that were mainly permeable to chloride. The ability to block the elicited chloride conductance with picrotoxin and, especially in vertebrate species, the alkaloid bicuculline (Nistri and Constanti, 1979), was an important pharmacological criterion for the identification of a GABA-mediated effect. This type of synaptic response became known as "classical" GABAergic inhibition. Later, bicuculline-insensitive receptors that mediated an inhibitory effect of GABA, which was mimicked by baclofen, but not by the classical GABA analog muscimol, were detected in vertebrates (Bowery, 1989). This receptor population was shown to be G-protein coupled, with activation eliciting changes in both calcium and potassium conductances (Wojcik et al., 1989). The identification of a second class of receptors which transduce GABA-mediated inhibition led to the present nomenclature, whereby the classical

receptor with an integral anion channel is termed the $GABA_A$ receptor, while the G-protein coupled receptor activated by baclofen is the $GABA_B$ receptor.

STRUCTURE OF THE $GABA_A$ RECEPTOR

Receptor Isolation

γ-Aminobutyric acid is the major inhibitory neurotransmitter in the mammalian central nervous system and the $GABA_A$ receptor mediates the majority of rapid inhibitory synaptic transmission. The density of the $GABA_A$ receptor is approximately 1 pmol per mg brain protein. In early attempts to purify the protein to homogeneity, many different non-denaturing detergents were employed to solubilize $GABA_A$ receptors from brain membrane preparations (for review, see Stephenson and Barnard, 1986). However, the best suited detergents for the preservation of receptor characteristics were shown to be the zwitterionic detergent, CHAPS (Sigel and Barnard, 1984) or the non-ionic detergent, ß-octylglucoside (Hammond and Martin, 1986; Bristow and Martin, 1987), used in the presence of protease inhibitors.

$GABA_A$ receptors have been successfully purified from mammalian brain by benzodiazepine affinity chromatography to achieve purification factors ranging from 2000 - 5000, but a yield of only 2 - 5% of the original starting binding activity (Sigel et al., 1983). It has been demonstrated that, despite the low recovery associated with membrane protein isolation, the pharmacological and physiological properties of the purified protein are representative of the population of receptors found in the membrane-

bound state (reviewed by Stephenson, 1988). Native $GABA_A$ receptors are known to contain multiple allosteric binding sites for many important pharmacological agents, including the clinically useful benzodiazepines and barbiturates (discussed in detail below). The purified material has been shown to contain (1) a high affinity binding site for the benzodiazepine ligands, (2) high and low affinity sites for the GABA agonist, [^{3}H]muscimol, and (3) positively co-operative effects of GABA and barbiturates on benzodiazepine binding affinity. The maintenance of some properties has been shown to be dependent on the presence of exogenous lipid throughout the purification process (Bristow and Martin, 1987). Affinity purified $GABA_A$ receptors have been reconstituted into lipid vesicles and shown to retain appropriate allosteric interactions and agonist-induced channel activation (Bristow and Martin, 1990; Thuynsma and Dunn, 1991; Dunn and Thuynsma, 1994). The kinetics of the $GABA_A$ receptor interactions have been studied with reconstituted preparations by entrapping a chloride sensitive fluorescent dye in liposomes and monitoring chloride conductances on a millisecond time scale with stopped flow fluorimetry (Dunn et al., 1989a, 1989b; Thuynsma and Dunn, 1991; Dunn and Thuynsma, 1994).

Molecular Properties of $GABA_A$ Receptor

The pattern obtained from denaturing polyacrylamide gel electrophoresis of purified $GABA_A$ receptors revealed a multi-subunit structure, with two major subunits evident (α of 53 kDa and ß of 57 kDa). The $GABA_A$ receptor was originally proposed to be a heterotetramer with a stoichiometry of $\alpha_2ß_2$, since the molecular weight of the

complex was predicted to range from 220,000 to 240,000 (see Stephenson, 1988). However, it is now generally accepted that the $GABA_A$ receptor is a pentameric complex comprised of a potentially diverse variety of subunit isoforms (see below).

The purified bovine receptor has been shown to be an acidic glycoprotein with an isoelectric point of 5.6 (Stephenson et al., 1986). The glycoprotein nature of the receptor was demonstrated by the specific binding of the receptor to lectin affinity columns (Gavish and Snyder, 1981; Stephenson and Olsen, 1983; Sigel et al., 1982) and carbohydrate staining of the purified receptor polypeptides following gel electrophoresis (Sigel and Barnard, 1984). In addition, the presence of glycosyl residues on the purified protein was confirmed by enzymatic deglycosylation of the polypeptides in the membrane-bound (Sweetnam and Tallman, 1986) and purified state (Mamalaki et al., 1987). The first information on the amino acid sequence of the receptor subunits was obtained from partial amino acid sequencing followed by molecular biological approaches. An α and a ß subunit were cloned using oligonucleotide probes designed from the partial sequence of proteolytic fragments generated from purified receptor preparations (Schofield et al., 1987). The primary sequences of these two subunits deduced from sequencing of their cDNAs showed that the subunits are homologous to each other and to the subunits of the nicotinic acetylcholine receptor.

The $GABA_A$ receptor is a member of the superfamily of ligand-gated ion channels (Schofield et al., 1987), that includes the nicotinic acetylcholine, glycine and $5HT_3$ receptors (Barnard, 1992). The ligand-gated ion channel superfamily is typified by receptors with the walls of the ion channel contributed from membrane-spanning regions

of each of the intrinsic subunits and the neurotransmitter binding site present on the same protein complex. By analogy to the well-characterized nicotinic receptor (Unwin, 1995) and from electron microscope images of purified porcine receptor (Nayeem et al., 1994), the $GABA_A$ receptor is believed to be a hetero-pentameric protein that spans the neuronal membrane to create a chloride conducting pore.

$GABA_A$ Receptor Subunit Structure

The homologous subunits which assemble to form the receptor - chloride channel complex are encoded by distinct, but related, genes (for review, see Burt and Kamatchi, 1991; Dunn et al., 1994). Although the precise stoichiometry of subunit isoforms that comprise native receptors remains unknown, to date six α, four ß, four γ, one δ, and two ρ subunit isoforms, plus splice variants for many of the genes, have been identified and classified by sequence homology. The functional significance of the vast diversity of $GABA_A$ receptor subunits is not known. It has been presumed that the complexity allows for such biological roles as tissue and age-dependent transcriptional control and functional control at the protein level.

All of the $GABA_A$ receptor subunits are of similar size, containing about 450 amino acids. An alignment of amino acids from the same subunit family (e.g. the six α-variants) typically shows 70 - 80% identity, while different family members exhibit only 30 - 40% identity. The receptor subunits are strongly conserved across species and the deduced protein sequences of the subunits identified to date share some highly similar domains considered to represent conserved structural motifs (for review, see Olsen and

Tobin, 1990). The common elements of the subunit structure include four putative membrane spanning domains, termed M1 through M4, composed of 22-23 amino acid residues with predominantiy hydrophobic character. The second membrane spanning region of each subunit has been implicated to line the integral chloride ion channel, based on both analogy to nicotinic receptors (reviewed by Claudio, 1989) and the high content of hydrophilic amino acids (serines and threonines) which would make the M2 helix conducive to chloride ion flow. Between the M3 and M4 domains, there is a large poorly conserved cytoplasmic loop that often contains consensus substrate sequences for several different protein kinases. These putative phosphorylation sites have been implicated to play a role in the regulation of $GABA_A$ receptor function and expression, although the physiological significance of receptor phosphorylation has not been determined (for review, see Macdonald and Olsen, 1994). Each subunit has a signal sequence that is presumed to be cleaved from the mature polypeptide, followed by a long hydrophilic extracellular amino-terminal domain that contains consensus sequences for potential asparagine-linked glycosylation; two sites in the α subunit and three in the ß and γ subunits. All subunits are believed to be glycosylated *in vivo*. The amino-terminal domain of each subunit also has a highly conserved pair of cysteine residues and, by forming a disulfide bond, these cysteines are presumed to form a fifteen residue ß-structural protein loop. It has not been firmly established if this structural loop plays a role in agonist binding to the $GABA_A$ receptor (Cockroft et al., 1990; Sumikawa and Gehle, 1992; Amin et al., 1994).

DIVERSITY OF $GABA_A$ RECEPTORS

Molecular cloning techniques have identified several $GABA_A$ receptor subunit isoforms thay may co-exist in various combinations to comprise multiple subtypes of native $GABA_A$ receptors. Although the subunit complement of native receptors remains undetermined, it is likely that different neuronal populations express different combinations of receptor subunits (for reviews, see Ludden and Wisden, 1991; Dunn et al., 1994; Macdonald and Olsen, 1994). To probe native receptor compositions in various areas throughout the mammalian brain, $GABA_A$ receptor gene products (messenger RNA and polypeptides) have been localized by *in situ* hydridization and immunocytochemistry. These methodological approaches have provided the first information about where certain subunit isoforms exist and the possible overlap or co-existence of more than one subunit isoform in the same neuron.

In Situ Hybridization

Although the existence of mRNAs encoding specific subunits do not necessarily confirm the functional expression of the polypeptide into $GABA_A$ receptors, the localization of these gene products has provided important clues for identification of receptor subtypes. The distribution of mRNAs for each subunit isoform in the central nervous system, as determined by *in situ* hybridization, is quite diverse (see Dunn et al., 1994). For example, the ρ-subunit is expressed only in retina (Cutting et al., 1991), whereas the isoforms of the α, ß, γ, and δ subunits show complex differences in regional

localizations, in addition to variable distributions throughout development. Several studies have suggested potential co-localization of particular receptor subunits, although with unknown stoichiometries, and some tentative major oligomeric assemblies have been proposed; eg. $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_x$, $\alpha_5\beta_1\gamma_x$, $\alpha_1\alpha_4\beta_2\delta_x$, and $\alpha_1\alpha_6\beta_2\delta_x$ (Wisden et al., 1992). It appears likely that a given α, ß, and γ subunit may combine with several different subunits to create a multitude of $GABA_A$ receptor subtypes.

Immunocytochemical Localization

The composition of subunit isoforms found in native $GABA_A$ receptors has also been probed with subunit-specific antibodies raised against purified receptors (Stephenson et al., 1986), specific subunits (Haring et al., 1985) and against synthetic peptides with sequences which are unique to specific subunits (Stephenson, 1992). Although it is uncertain if all $GABA_A$ receptors that are assembled become inserted into the neuronal membrane or if all inserted receptors are functional, immunological characterization studies have provided much information on $GABA_A$ receptor structure. Immunological approaches with subunit-specific antibodies have been used for the identification of receptor subunits on Western blots and the localization of certain isoforms in brain sections, as well as for the analysis of subunit composition by immunoprecipitation and immunoaffinity chromatography. Antibodies raised against the original receptor subunits identified (now known as α_1 and β_1) were found to stain several polypeptides in purified receptor preparations (Kirkness and Turner, 1988; Fuchs et al., 1990). This cross-reactivity is now believed to be the result of other isoforms of α and ß subunits present

in isolated receptors, as the polypeptide gene products for α_1, α_2, α_3, α_5, β_2, β_3, γ_2, and δ have been identified by western blotting techniques (see Macdonald and Olsen, 1994).

In immunoprecipitation studies, α-subunit specific antibodies have recognized and precipitated variable amounts of receptor in different brain regions. While these receptors have been shown to contain primarily a single α-subunit isoform (Duggan and Stephenson, 1990), some apparently contain multiple α-subunit isoforms (Duggan et al., 1991, McKernan et al., 1991, Endo and Olsen, 1993, Pollard et al., 1993). Essentially all of the $GABA_A$ receptors from bovine cortex purified by benzodiazepine affinity chromatography are immunoprecpitated by antibodies recognizing ß subunits (Endo and Olsen, 1992). Using purified receptor preparations, several studies have found that antibodies to the β_3 subunit immunoprecipitate about 30-50% of receptors (Pollard et al., 1991), the γ_2-subunit specific antibodies precipitate 40 - 75% (Benke et al., 1991a; Stephenson et al., 1990) and δ subunit specific antibodies precipitate about 20 - 30% (Benke et al., 1991b, Mertens et al., 1993). However, the interpretation of these immunoprecipitation studies may be compromized if receptor aggregation occurs during the purification process (Pollard et al., 1993).

A diverse pattern of distribution for subunit isoforms throughout the brain became apparent from early studies using immunohistochemical localization. At least five oligomeric $GABA_A$ receptor subtypes were required to explain the differential localization of only α_1, α_3, $\beta_{2/3}$ and γ_2 immunoreactivities (Fritschy et al., 1992). Taken together, these studies suggest that one to two dozen $GABA_A$ receptor subtypes are present in the central nervous system, with the potential for some to co-exist in a single neuronal cell

type. Oligomeric $GABA_A$ receptors have also been shown to occur in glial cells (MacVicar et al., 1989), which further complicates the attempts to identify the subunit complement for neuronal $GABA_A$ receptors.

Heterologous Expression

Heterologous expression studies of $GABA_A$ receptor subunits in *Xenopus* oocytes and mammalian cells have been undertaken to determine the ligand binding characteristics and functional attributes of receptors assembled from various subunit isoforms (see Dunn et al., 1994; Macdonald and Olsen, 1994). It has been found that functional $GABA_A$ receptor subtypes can be produced from varied combinations of subunits, but the characteristics of expressed receptors appear to depend on the cell type used for expression. For example, expression of α, ß, and γ subunits in *Xenopus* oocytes has shown that receptors form from monomeric, dimeric and trimeric combinations, although with different levels of expression (Schofield et al., 1987; Blair et al., 1988; Shivers et al., 1989; Sigel et al., 1990). In contrast, for high efficiency expression of functional receptors in HEK 293 fibroblasts, it has been shown that co-expression of subunits is required, with isoform combinations such as $\alpha_1ß_2$, $\alpha_1\gamma_2$ and $\alpha_1ß_2\gamma_{2s}$ (Verdoorn et al., 1990). While the subunit combinations created by heterologous cell expression may not accurately represent native $GABA_A$ receptors, since interpretation may be compromised by a number of potential difficulties (see Dunn et al., 1994), these data suggest that there is ordered assembly of subunits to produce functional receptors. Another important concept realized from studies on recombinantly expressed receptors is that specific roles

may be attributed to individual subunit classes and interactions between different subunit classes. The contribution that different subunits and their isoforms can make to the pharmacological profile of a $GABA_A$ receptor, as determined by heterologous expression studies, will be discussed in more detail below.

PHARMACOLOGY OF THE $GABA_A$ RECEPTOR

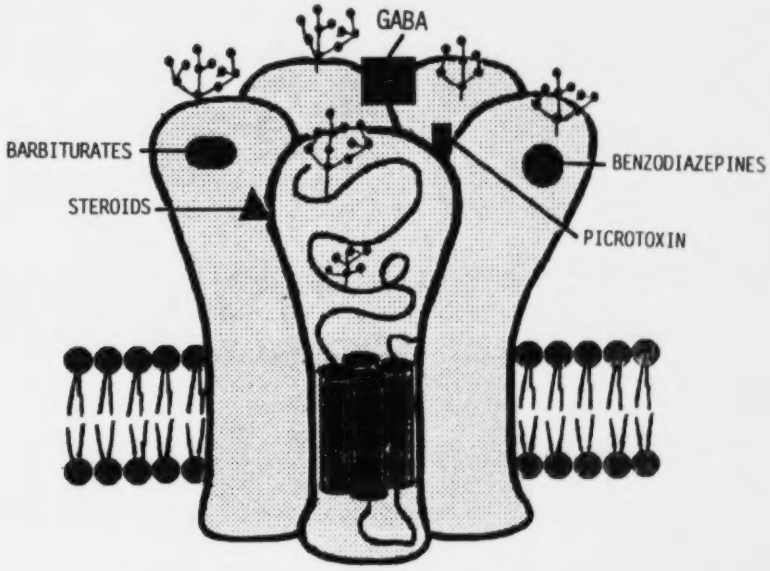


Figure 1.1 General schematic of the $GABA_A$ receptor - chloride channel complex.

Overview

A multiplicity of neuroactive drugs have been shown to interact specifically with the $GABA_A$ receptor - chloride channel complex to modulate inhibitory neurotransmission throughout the brain. In addition to binding sites for GABA and its analogues, the

$GABA_A$ receptor carries distinct sites for many important pharmacological agents, including the benzodiazepines, barbiturates, picrotoxin, some naturally-occurring steroids, general anaesthetics and possibly alcohol (for reviews, see Sieghart, 1992; Dunn et al., 1994; Smith and Olsen, 1994). While both the benzodiazepines and barbiturates have been shown to enhance GABA-mediated chloride currents (Choi et al., 1977, Macdonald and Barker, 1978; 1979), this modulation occurs through distinct allosteric regulatory sites on the receptor complex (Study and Barker, 1981; Twyman et al., 1989a; Olsen, 1981). The anaesthetic steroids and progesterone metabolites also potentiate GABA mediated neuronal inhibition (Majewska et al., 1986). GABA-elicted currents are reduced by the drugs bicuculline, picrotoxin and methyl 6,7-dimethoxy-4-ethyl-ß-carboline-3-carboxylate (DMCM), all interacting with the receptor via different sites.

The GABA Binding Site

The binding of radiolabelled GABA to receptors *in vitro* was first described by Zukin et al., 1974 and Enna and Snyder, 1975, and within the next few years, GABA binding was shown to meet many of the criteria for receptor identification, including inhibition by the analogues which were known to be functional agonists and antagonists at GABA synapses (Greenlee et al., 1978; Krogsgaard-Larsen et al., 1979). Under conditions designed to prevent sodium-dependent membrane uptake, to remove endogenous GABA and to stabilize receptors, characteristics such as low density, appropriate affinity, and specific subcellular and tissue localization were demonstrated (Olsen, 1982; Stephenson, 1988). At equilibrium, the binding of GABAergic agonists

to brain membranes from vertebrates of various ages, in many brain regions and subcellular fractions, is heterogeneous. [^{3}H]GABA binding is best represented by two populations of sites with different affinity (K_D values of 10 - 20 nM and 100 nM - 1 μM), both having receptor-like pharmacological specificity (Olsen et al., 1981). The sites with lower affinity are presumed to be associated with physiological function, since micromolar concentrations of agonists are required to induce chloride flux and to modulate the binding of benzodiazepines (reviewed by Fischer and Olsen, 1986). The high affinity sites are predicted to reflect the receptor in an inactivated and desensitized state. It has yet to be determined whether the high and low affinity sites for GABA represent binding to distinct sites or to interconverting conformational states of the receptor (Agey and Dunn, 1989). To date, no compound has been identified which selectively interacts with a subset of the $GABA_A$ binding sites. The plant derived convulsant, bicuculline, competitively antagonizes GABA-induced chloride current (Macdonald et al., 1989a), most likely by direct competition with GABA for binding to the receptor. The binding of [^{3}H]bicuculline to mammalian brain membranes appears to display a relative selectivity for the low affinity GABA binding sites (Olsen and Snowman, 1983).

The involvement of particular amino acids in the binding of GABA and other agonists has been studied using protein modification techniques. Treatment of brain membrane preparations or purified $GABA_A$ receptors with arginine modifying reagents such as 2,3-butanedione or phenylglyoxal resulted in a loss of binding sites, suggesting that arginine residues play a role in [^{3}H]muscimol binding (Widdows et al., 1987).

Histidine and tyrosine residues, when modified by diazotized sulfanilate, resulted in a loss of low, but not high, affinity [^{3}H]GABA binding sites and a parallel loss of the allosteric modulation of benzodiazepine binding by GABA (Burch et al., 1983).

In addition, while the specific chemical reaction that generates the photolabile reactive species is unresolved, photoaffinity labelling of $GABA_A$ receptor agonist sites in purified preparations with [^{3}H]muscimol has shown the major site of photolabelling to be associated with the ß subunit (Casalotti et al., 1986; Deng et al., 1986; Fuchs and Sieghart, 1989). However, [^{3}H]muscimol photoaffinity labelling has been reported to occur on the α-subunits (Bureau and Olsen, 1990), suggesting that these subunits are also involved in agonist binding. Using molecular biological approaches, a single point mutation of phenylalanine at residue 64 in the extracellular amino-terminal domain of the rat α_1 subunit resulted in a decrease in agonist and antagonist affinities for recombinantly expressed $\alpha_1ß_2\gamma_2$ receptors (Sigel et al., 1992). Subsequently, this Phe residue (found at homologous position 65 in bovine receptors) was shown to be photoaffinity labelled by [^{3}H]muscimol (Smith and Olsen, 1994), suggesting that it is most likely positioned near a GABA binding site. Homologous segments of protein sequence from this domain are found in all α-subunits and in the γ_2 and δ, but not in the ß subunits. In the $ß_2$ subunit, site directed mutagenesis has been employed to identify two homologous domains located between the disulfide loop and the first transmembrane domain (Tyr157-Gly158-Tyr159-Thr160 and Thr202-Gly203-Ser204-Tyr205) that are involved with functional activation of $GABA_A$ receptors by GABA (Weiss and Amin, 1993).

Considered together, this evidence suggests that agonist binding sites occur at the

interface(s) between subunits and/or that both α and ß subunits, as well as others, carry binding sites for GABA/muscimol.

The Benzodiazepine Binding Site

The first clinically useful benzodiazepine, chlordiazepoxide hydrochloride, was discovered in 1957 after a search for compounds with sedative and anxiolytic properties. These drugs remain amongst the most frequently prescribed of all psychoactive drugs for their usefulness as anxiolytics and sedative-hypnotics, although they are also potent anticonvulsants and muscle relaxants (for review, see Martin, 1990). However, the mechanism of action by which the benzodiazepines elicit their effects was not conclusively established for several years following their introduction into clinical practice.

The first implication came from studies of the cat spinal cord, where diazepam was found to increase the magnitude and duration of the dorsal root potential (DRP) (Schmidt et al., 1967). Although the transmitter released at this synapse was predicted to be GABA (Eccles et al., 1962, 1963), this was not confirmed until several years later (Levy, 1977). However, in parallel studies, it was the GABAergic transmission at this synapse that was used to demonstrate that GABA and the $GABA_A$ receptor was integral to the mechanism of action of the benzodiazepines (Polc et al., 1974). Subsequently, the benzodiazepines were shown to modulate GABA-mediated inhibition in various regions of the mammalian central nervous system; the cuneate nucleus (Polc and Haefely, 1976), hippocampal pyramidal cells (Wolf and Haas, 1977) and the cerebral cortex (Raab and

Gumnit, 1977). The action of the benzodiazepines on GABAergic synaptic transmission was shown to be dose-dependent and to potentiate the effects of a submaximal stimulus, giving a parallel left-ward shift in the dose-response curve for GABA, but not altering the maximal response (Macdonald and Barker, 1978; Choi et al., 1981).

Several studies have now firmly established that benzodiazepines produce a facilitation of $GABA_A$ receptor mediated transmission at both pre- and post- synaptic sites in the mammalian central nervous system (reviewed by Haefely and Polc, 1983). It is also known that subsets of benzodiazepine ligands exist, where flunitrazepam and diazepam are full "classical" agonists and ligands such as clonazepam and chlordiazepoxide are partial agonists (Chan et al., 1985). Some benzodiazepine compounds act as inverse agonists and shift the GABA dose response curve to the right, thereby reducing the inhibitory response elicited by GABA (Kemp et al., 1987). The imidazobenzodiazepine, Ro15-4513 (ethyl-8-azido-5-6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5a][1,4]-benzo-diazepine-3-carboxylate), is a partial inverse agonist. Additionally, benzodiazepines such as Ro15-1788, also known as flumazenil, antagonize the effects of both classical and inverse agonists.

Electrophysiological studies using fluctuation analysis implied that the classical benzodiazepines enhance the actions of GABA at the $GABA_A$ receptor by increasing the frequency of chloride channel opening (Study and Barker, 1981). This was recently confirmed by single channel recording techniques which showed that benzodiazepines increase the channel opening frequency and burst activity, without altering mean open time or conductance (Vicini et al., 1987; Twyman et al., 1989a). It has been suggested

that diazepam produces these effects on channel activity by increasing the apparent association rate of GABA, without altering channel gating (Rogers et al., 1994). Alternatively, it has also been proposed that benzodiazepine modulation of GABA-mediated inhibition is via changes in gating characteristics of the $GABA_A$ receptor channel (Mody et al., 1994).

The binding of radioligand benzodiazepines to brain membrane preparations was first demonstrated in 1977, when saturable, specific, high affinity binding sites for [^{3}H]diazepam were found differentially distributed throughout the brain (Möhler and Okada, 1977; Squires and Braestrup, 1977). The structure-activity relationships at this binding site correlated well with the rank order of efficacy for the pharmacological activity of benzodiazepines, suggesting that the site was indeed the receptor through which these ligands elicited their actions. It is now clear that several structurally diverse compounds interact with high affinity at the benzodiazepine binding site. Currently, three radiolabelled benzodiazepine ligands: [^{3}H]flunitrazepam, [^{3}H]Ro15-4513 and [^{3}H]flumazenil (an agonist, partial inverse agonist and antagonist, respectively) are the most widely used ligands for the study of benzodiazepine binding sites.

Autoradiographic studies have demonstrated that [^{3}H]flunitrazepam and [^{3}H]GABA or [^{3}H]muscimol binding sites are co-localized throughout the mammalian brain (Möhler and Okada, 1977), with high receptor densities found in cortex, cerebellum and hippocampus and lower levels in basal ganglia, thalamus and the hind-brain (Young and Kuhar, 1979; Penney et al., 1981). In the periphery, the outer membrane of mitochondria possess [^{3}H]diazepam binding sites that are distinct from central-type

GABA/benzodiazepine receptors and are thought to be involved in steroidogenesis (Braestrup and Squires, 1977; Anholt et al., 1986).

The benzodiazepines interact with the $GABA_A$ receptor via an allosteric mechanism to modulate inhibition throughout the central nervous system. A functional link between the benzodiazepines and the $GABA_A$ receptor was first implicated by the observation that the binding of radiolabelled benzodiazepines is enhanced by co-incubation with GABA and that this stimulation is antagonized by bicuculline (Martin and Candy, 1978; Tallman et al., 1978; Briley and Langer, 1978). Further, the binding of benzodiazepines is stimulated by certain inorganic anions, notably chloride (Martin and Candy, 1980; Costa et al., 1979). Benzodiazepine binding is not displaceable by GABA agonists or antagonists, and the binding of GABA analogues cannot be displaced from their binding site by the benzodiazepines. On the basis of their sensitivity to the triazolopyridazine, CL-218872, benzodiazepine binding sites were originally classified pharmacologically into two major groups. CL-218872 displaces classical benzodiazepine agonist binding from cerebellar $GABA_A$ receptor populations with an apparently higher affinity than for receptors in the hippocampus (Squires et al., 1979). The cerebellar receptors with high affinity for CL218872 are referred to as type I sites, while the population in the hippocampus that exhibits a lower affinity for CL218872 are termed type II (Martin et al., 1983). More recently available molecular biological approaches utilizing recombinantly expressed receptor systems have demonstrated that the α_1 subunit determines type I, while the α_2, α_3 and α_5 isoforms confer type II, benzodiazepine pharmacology (Pritchett et al., 1989a; Pritchett and Seeburg, 1990). In addition, site-

directed mutagenesis of α_3 Glu225 to α_1 Gly225 results in a functional switch from type II to type I benzodiazepine pharmacology (Pritchett and Seeburg, 1991).

Certain benzodiazepines, such as flunitrazepam, clonazepam and Ro15-4513, can be used as photoaffinity labelling reagents and have provided useful tools for the study of the molecular nature of the $GABA_A$ receptor binding sites (Battersby et al., 1979; Sieghart and Karobath, 1980; Möhler et al., 1980; Sieghart et al., 1987a, 1987b). Upon irradiation with ultraviolet light, these ligands covalently incorporate into the $GABA_A$ receptor. The specificity of the photoaffinity labelling reaction for the $GABA_A$ receptor was confirmed by a GABA-stimulated enhancement of labelling and inhibition of this enhancement by bicuculline (Sieghart and Karobath, 1980). Although [^{3}H]flunitrazepam specifically photoincorporates into about one quarter of the total sites available for 'reversible' binding, the photoaffinity labelling reaction results in the irreversible inactivation of the remaining binding sites such that benzodiazepines will no longer bind with high affinity (Möhler et al., 1980; Thomas and Tallman, 1981). While the inactivation of binding sites is presumably due to a change in receptor conformation, the mechanism by which this effect occurs is unknown. [^{3}H]Flunitrazepam, like [^{3}H]muscimol which has also been used as a photoaffinity label (see above), is not a conventional photoactive ligand and the specific photolabile group that generates a reactive species has not been identified.

Denaturing gel electrophoresis of crude brain membrane preparations, photoaffinity labelled with [^{3}H]flunitrazepam, first showed that the label was associated with a major 51 kDa protein (Möhler et al., 1980). With purified $GABA_A$ receptors,

[^{3}H]flunitrazepam was shown to photoaffinity label the 52 kDa α-subunit (Sigel and Barnard, 1984). However, more detailed studies of photoaffinity labelled receptors resolved by gel electrophoresis indicated heterogeneity in the labelling patterns of $GABA_A$ receptors from various brain regions and different stages in development, as well as heterogeneity in the pattern of labelled fragments generated from proteolytic cleavage by trypsin (Sieghart and Drexler, 1983). It was subsequently shown that [^{3}H]flunitrazepam labelled three α-subunit isoforms with apparent molecular weights of 51, 53 and 59 kDa (Fuch et al., 1988) and subunit-specific antibodies have demonstrated that these proteins correspond to the α_1, α_2, and α_3 subunits, respectively (Stephenson et al., 1989, Fuchs et al., 1990).

The site on the α-subunit(s) for covalent attachment of the [^{3}H]flunitrazepam photolabel has been limited to lie within the large extracellular amino-terminal domain (Czajkowski and Farb, 1986; Schmitz et al., 1989). To identify the specific binding site, fragments that were generated by *Staphylococcus aureus* V8 protease cleavage of purified $GABA_A$ receptors, photoaffinity labelled with [^{3}H]flunitrazepam, have been partially sequenced and indicate that the site most likely occurs within amino acid residue 8 - 297 of the α_1 sequence (Olsen et al., 1991). Another study which probed peptide fragments of photoaffinity labelled receptors with sequence-specific antibodies concluded that the [^{3}H]flunitrazepam label does not lie within the amino acid sequences 1 - 58 or 149 - 429 of the α_1 subunit (Stephenson and Duggan, 1989). Taken together, these results would suggest that the site of labelling lies within residues 59 - 149 of the α_1 subunit. While the evidence for the α-subunits being the major site of photolabelling for [^{3}H]-

flunitrazepam is strong, these subunits are not the sole determinants of benzodiazepine binding. In receptor expression studies, the presence of a γ-subunit has been shown to be necessary for the binding of benzodiazepines and their modulation of $GABA_A$ receptor responses (Pritchett et al., 1989b). In addition, a point mutation of the γ-subunit at Thr142 affects benzodiazepine modulation of $GABA_A$ receptor responses (Mihic et al., 1995) and data from a photoaffinity labelling study suggested some [^{3}H]flunitrazepam label may incorporate with the γ-subunit (Stephenson and Duggan, 1989).

Ro15-4513, an azide derivative of flumazenil, has been used as a photoaffinity reagent and covalently labels all available benzodiazepine binding sites (Sieghart et al., 1987b). This partial inverse agonist is known to bind competitively to sites that are sensitive to agonists and antagonists, as well as to a sub-population of $GABA_A$ receptors that are insensitive to the classical benzodiazepine agonists. These "diazepam-insensitive" $GABA_A$ receptors are found mainly in the cerebellum (about 20% of sites) and to a very minor extent (less than 2.5% of sites) in the cerebral cortex (Sieghart et al., 1987b; Turner et al., 1991). The diazepam-insensitive binding sites correlate with the presence of α_4 and α_6 subunits in the $GABA_A$ receptor complex (Lüddens et al., 1990; Wieland et al., 1992). The $GABA_A$ receptor sites that are photoaffinity labelled by [^{3}H]Ro15-4513 appear to be associated with the same subunits that are labelled by [^{3}H]flunitrazepam. Electrophoretic patterns of membrane homogenates from cerebral cortex and hippocampus, photoaffinity labelled with [^{3}H]Ro15-4513, have shown major labelled proteins of 51 and 55 kDa, while preparations from the cerebellum indicate an additional 57 kDa labelled protein. The 57 kDa cerebellar protein is not photolabelled

by [^{3}H]flunitrazepam and is presumed to represent the diazepam-insensitive population of benzodiazepine binding sites.

Chemical modification techniques have been used to provide some valuable information on the importance of certain amino acid residues in benzodiazepine ligand binding. Several groups have investigated the effects of reacting $GABA_A$ receptor preparations with diethyl-pyrocarbonate (Burch and Ticku, 1981; Sherman-Gold and Dudai, 1981; Burch et al., 1983; Maksay and Ticku, 1984). Results from these studies have implicated histidine residues to be involved in the binding of benzodiazepines and ß-carbolines to the $GABA_A$ receptor. Although diethyl-pyrocarbonate (DEP) is known to react with histidine and tyrosine residues, both benzodiazepine binding and DEP inactivation display a pH dependency consistent with the involvement of histidine residues (Lambolez and Rossier, 1987; Lambolez et al., 1989; Maksay et al., 1991) The binding of [^{3}H]Ro15-4513 to both the diazepam-sensitive and diazepam-insensitive $GABA_A$ receptor populations (described above), associated with α_4 and α_6 subunits (Luddens et al., 1990; Wieland et al., 1992), is not affected by DEP receptor modification (Binkley and Ticku, 1991; Maksay, 1992; Uusi-Oukari, 1992). Thus, the binding of the partial inverse agonist does not involve the histidine residue(s) that is/are crucial for the binding of the other benzodiazepine agonists and antagonists.

A histidine residue at position 102 in the α_1 subunit has been shown, by site directed mutagenesis, to be essential for high affinity agonist binding. The α_4 and α_6 subunits possess an arginine residue in the equivalent position (Wieland et al., 1992; Wisden et al., 1991, Korpi et al., 1993). By treating cerebellar membranes with the

arginine-modifying reagent, 2,3-butanedione (Maksay, 1992), the possible importance of arginine residues in [^{3}H]Ro15-4513 binding has been investigated. Inhibition of [^{3}H]Ro15-4513 binding to the diazepam-insensitive site was slightly more sensitive to 2,3-butanedione than was the diazepam-sensitive site.

Cysteine/cystine residues have also been implicated to play a structural role in the binding of benzodiazepines to the $GABA_A$ receptor. The binding affinity for [^{3}H]diazepam and for the GABA analogue, [^{3}H]muscimol, was reduced by treatment of brain homogenates with the sulfhydryl alkylating agent, iodoacetamide, or the disulfide reducing agent, ß-mercaptoethanol (Marangos and Martino, 1981). However, more recent investigations re-examined these effects and found that iodoacetamide treatment of bovine brain membranes had little effect on the binding of flunitrazepam, but reduction of the membranes with dithiothreitol prior to treatment with excess iodoacetamide markedly reduced the affinity of flunitrazepam binding (Otero de Bengtsson et al., 1993).

The Picrotoxin/TBPS Binding Site

The plant convulsant picrotoxin (the active ingredient is picrotoxinin) and some bicyclophosphate cage compounds such as tert-butylbicyclophosphorothionate (TBPS) antagonize GABA-induced chloride currents. These agents do not inhibit GABA receptor binding, i.e., the inhibition of the currents elicited by GABA is noncompetitive and occurs via a site distinct from that for GABA (Olsen, 1981). These convulsants do not displace high affinity benzodiazepine binding, although certain benzodiazepines (Ro5-3663, Ro5-4864) are convulsant due to their interaction at this site rather than with the

benzodiazepine site (Olsen, 1981). The binding sites have been identified by [^{3}H]α-dihydropicrotoxinin (DHP) and [^{35}S]TBPS binding (Squires et al., 1983) and seem to be closely associated with the integral chloride channel of the $GABA_A$ receptor. The convulsant compounds that bind to the DHP/TBPS site appear to reduce chloride conductance by a direct mechanism of sterically hindering chloride entry into the ion channel (Gee, 1988). However, the binding may allosterically prevent the conformational change of the receptor protein required for chloride channel opening upon GABA binding (Olsen and Tobin, 1990; Delorey and Olsen, 1992). The exact nature of the binding has therefore not been elucidated. The binding affinity of [^{35}S]TBPS is reduced by allosteric interactions with $GABA_A$ receptor agonists and enhanced by some receptor antagonists, like convulsant ß-carbolines, through specific interactions with the benzodiazepine sites. Electrophysiological findings from single channel recordings suggest that picrotoxin may alter the intrinsic gating of the channel once GABA is bound, so that the rates of channel opening into various states are altered (Twyman et al., 1989b).

The Barbiturate Binding Site

Sedative hypnotic, anticonvulsant and anaesthetic barbiturates, such as pentobarbital or secobarbital, potentiate GABA-mediated chloride conductances as measured by electrophysiological methods or by radiotracer ion flux, by a mechanism distinct from that of the benzodiazepines (for review, see Olsen, 1982). Barbiturates both enhance the actions of GABA and mimic GABA by causing receptor activation and

channel opening in the absence of GABA (Nicoll et al., 1975). Furthermore, barbiturates have been shown to shift the GABA dose response curve to the left, and at higher concentrations, increase the maximum response (Gallagher et al., 1981). The demonstration of direct binding of barbiturates to their recognition sites has not been possible because of their low affinity for these sites. However, *in vitro*, the barbiturates have been shown to enhance $GABA_A$ receptor affinities (Olsen and Snowman, 1982), in a manner that correlates with their order of potency as anaesthetics and hypnotics. The mechanism for these effects is presumably by some allosteric interaction within the receptor complex, since the barbiturates enhance the binding of GABA and benzodiazepine agonists, while inhibiting the binding of GABA antagonists and benzodiazepine inverse agonists (Olsen et al., 1986). It is the binding of GABA to low affinity sites that is affected by the barbiturates (Yang and Olsen, 1987). Strong correlations between the structure-activity relationships for enhancement of GABA receptor function and the allosteric binding interactions have been demonstrated for many barbiturate compounds (Olsen, 1981, 1987). While the relationship of barbiturate-induced increases in GABA binding to channel activation is unclear, these interactions are dependent upon physiological concentrations of chloride or other anions that can permeate the receptor channel.

Electrophysiological experiments using fluctuation analysis suggest that barbiturates increase the average open duration of the $GABA_A$ receptor chloride channel, without altering channel conductance (Barker and McBurney, 1979; Study and Barker, 1981). Subsequently, this effect was demonstrated directly from single-channel

recordings of $GABA_A$ receptor currents enhanced by barbiturates, where the average channel open duration was increased, but conductance or opening frequency was unaffected (Mathers and Barker, 1981; Jackson et al., 1982; Macdonald et al., 1989b, Twyman et al., 1989b). However, another electrophysiological study presented data from open duration frequency histograms that suggested the barbiturates do not alter the open duration time constants (Macdonald et al., 1989), but rather reduce the relative proportion of channel openings with short durations and increase the proportion with longer durations. These results imply that the barbiturates alter the intrinsic gating of the channel once GABA is bound.

The barbiturates allosterically inhibit the *in vitro* binding of the plant convulsant, picrotoxin (Ticku et al., 1978) and studies of the channel gating characteristics indicate that picrotoxin acts in a reciprocal manner to the barbiturates, in that channel burst duration is decreased (Twyman et al., 1989b). Picrotoxin is known to competitively displace the cage convulsant TBPS from its specific high affinity binding sites on brain membranes in a simple competitive fashion (Squires et al., 1983). However, the interaction of the barbiturates with the TBPS binding sites is very complex (Trifiletti et al., 1984), suggesting that the barbiturates interact with a site different from that of picrotoxin. It appears that the barbiturates enhance GABA-mediated chloride conductance by interacting with a binding site distinct from that of GABA agonists, benzodiazepines or TBPS. In addition, the pharmacological action of barbiturates on $GABA_A$ receptors is varied throughout different brain tissues, suggesting the possible existence of $GABA_A$ receptor subtypes with different barbiturate binding properties.

The Steroid Binding Site

Evidence from multi-disciplinary studies suggests that the action of anaesthetic steroids such as alfaxalone and the sedative-hypnotic-anxiolytic metabolites of progesterone and desoxycortone result from potentiation of $GABA_A$ receptor mediated transmission (Harrison and Simmonds, 1984; Puia et al., 1990). These steroids and their derivatives enhance GABA-stimulated chloride conductance in brain apparently by prolonging the open time of the chloride channel (Peters et al., 1988). In addition, these compounds enhance the binding affinity of the GABA analogue, [^{3}H]muscimol, and the classical benzodiazepine agonist, [^{3}H]flunitrazepam, but inhibit the binding of [^{35}S]TBPS to the receptors (Gee, 1988; Schumacher and McEwen, 1989). It appears that barbiturates potentiate steroid-activated transmembrane currents (Peters et al., 1988) and that the interaction of [^{35}S]TBPS and [^{3}H]flunitrazepam binding with steroids was inconsistent with direct competition at common sites (Gee, 1988). Taken together, these experiments implicate a separate site of action for the steroids that is distinct from the sites for binding of GABA, benzodiazepines, barbiturates and DHP/TBPS. It has also been suggested that the effects of steroids may be mediated by specific interactions with the membrane lipid - $GABA_A$ receptor protein interface, due to their high lipophilic nature and evidence that phospholipids can specifically bind steroids (Gee, 1988).

In electrophysiological experiments, steroids have been shown to increase the average channel open time and burst durations by increasing the frequency of opening and the occurrence of longer open states (Twyman and Macdonald, 1992). The differential effect of steroids and barbiturates on the frequency of channel opening seems

to corroborate the belief that these compounds interact with distinct receptor sites. However, the mechanisms for the observed prolongation of the open channel by steroids is similar to that described for barbiturates, suggesting that the steroids and barbiturates may regulate the channel through at least one common effector mechanism (Macdonald and Olsen, 1994).

Other Binding Sites

The $GABA_A$ receptor contains binding sites for several other modulatory compounds, such as avermectin, Ro5-4854, zinc, chloride and possibly ethanol (reviewed by Sieghart, 1992). Avermectin B_{1a}, a lactone with potent insecticidal and anthelmintic actions, has been shown to specifically affect the interaction of the $GABA_A$ receptor with agonists and antagonists in a manner which indicates a close association of a distinct avermectin binding site with the receptor complex (Drexler and Sieghart, 1984). The prototypic peripheral benzodiazepine site ligand, Ro54864, interacts with the $GABA_A$ receptor at micromolar concentrations and acts as a potent convulsant. Several studies have suggested the presence of a Zn^{2+} binding site at some $GABA_A$ receptors and GABAergic neurons have been shown to be selectively inhibited by this divalent cation. The Zn^{2+} site appears to be localized extracellularly and to be distinct from the other receptor sites previously discussed. Finally, ethanol has been shown to exhibit properties consistent with an action at GABAergic synapses and $GABA_A$ receptors (Celentano et al. 1988; Ticku, 1990), although an interaction with a specific $GABA_A$ receptor site remains inconclusive.

CHAPTER 2

The Major Site of Photoaffinity Labelling of the $GABA_A$ Receptor by [^{3}H]Flunitrazepam is Histidine 102 of the Alpha Subunit

A version of this chapter has been submitted for publication. Duncalfe, L.L., M.R. Carpenter, L.B. Smillie, I.L. Martin and S.M.J. Dunn, 1995, J. Biol. Chem.

INTRODUCTION

Many psychoactive drugs have been shown to interact specifically with the $GABA_A$ receptor complex to modulate inhibitory neurotransmission throughout the brain (see Chapter 1). Because of the clinical usefulness of the benzodiazepines as anxiolytics and sedative-hypnotics, their interaction with the $GABA_A$ receptor has been extensively studied. Particular interest has focused on the identification of the protein domains involved in the interaction of benzodiazepines with the $GABA_A$ receptor. Using multi-disciplinary approaches, several groups have identified structural features of subunit isoforms that are important for ligand recognition and for the modulatory effects of the benzodiazepines (reviewed by Smith and Olsen, 1995). To date, site-directed mutagenesis has identified the amino acids Gly225 of the α_1 subunit (Pritchett and Seeburg, 1991), His101 of the α_1 subunit (Wieland et al., 1992) and Thr142 of the γ_2 subunit (Mihic et al., 1995) as residues which play a role in conferring the differential binding affinities/efficacies of benzodiazepine ligands for the $GABA_A$ receptor. Biochemical approaches have shown that the site of photoaffinity labelling by the classical agonist, [^{3}H]flunitrazepam, is associated with the α-subunit of the $GABA_A$ receptor (Fuchs et al., 1988; Stephenson et al., 1990), within the large extracellular amino-terminal domain (Czajkowski and Farb, 1986; Schmitz et al., 1989). Partial sequences of proteolytic fragments from photoaffinity labelled receptors have indicated that the [^{3}H]flunitrazepam site occurs within amino acid residues 8 - 297 of the α_1 subunit (Olsen et al., 1991) and, using subunit specific antibodies, the site has been

predicted to occur within residues 59 - 158 of the α_1 subunit sequence (Stephenson and Duggan, 1989). In addition, the [^{3}H]flunitrazepam photoaffinity labelled peptides generated by hydroxylamine cleavage have been mapped to known $GABA_A$ receptor sequences to limit the site of photolabelling to within amino acids 1 - 103 of the α subunit (discussed in Chapter 3). These studies, considered together, limit the predicted site of labelling to within residues 59 - 103 of the α_1 subunit or within homologous segments of other α subunit isoforms.

In the present study, immunoprecipitation and HPLC techniques have been used to purify a [^{3}H]flunitrazepam photoaffinity labelled peptide that was generated by cyanogen bromide cleavage of labelled $GABA_A$ receptors from bovine cerebral cortex. It is shown by peptide mapping and microsequence analysis that the major site of [^{3}H]flunitrazepam photoincorporation by the $GABA_A$ receptor is likely to be the amino acid His102 of the bovine α_1 subunit.

MATERIALS AND METHODS

Preparation of Membranes from Bovine Cerebral Cortex

Adult bovine brain was obtained from a local slaughterhouse. The cerebral cortex was dissected out and it was immediately frozen on dry ice and stored at -80°C. Brain membranes were prepared from approximately 100 g of partially thawed cortex that was chopped with a chilled razor blade and homogenized in 10 volumes of 100 mM Tris-HCl pH 7.2, 0.3 M sucrose, 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.1

mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml soybean trypsin inhibitor and 0.02% NaN_3. The first homogenization was performed at 4°C with a Waring blender operated at high speed for four 20-s periods. The mixture was homogenized a second time, at 4°C and on ice, using a Virtis 45 at setting 30 for two 20-s periods and then centrifuged at 2500 rpm in a Sorvall GSA rotor for 10 min at 4°C. The supernatant was filtered through eight layers of cheese cloth and centrifuged at 40,000 rpm in a Beckman type 45 Ti rotor for 45 min at 4°C (150,000*g*). The pellets were resuspended with the Virtis homogenizer at setting 30 for four 20-s periods in 100 mM Tris-HCl pH 7.2, 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor and 0.02% NaN_3 and the mixture was re-centrifuged for 45 min at 40 000 rpm. The final resuspension of the pellets was in 20 mM Tris-citrate pH 7.4, 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.3 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml bacitracin and 0.02% NaN_3 at a protein concentration of 10-15 mg/ml, as assayed by the method of Bradford (1976). The membranes were aliquoted, frozen in liquid nitrogen and stored at -80°C.

Photoaffinity Labelling of $GABA_A$ Receptors from Bovine Cerebral Cortex with [^{3}H]Flunitrazepam

For the photoaffinity labelling reaction, membrane aliquots were thawed and diluted to a final protein concentration of 2 mg/ml in 20 mM Tris-citrate pH 7.4 buffer containing 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.3 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml bacitracin and 0.02% NaN_3. [N-Methyl-

^{3}H]flunitrazepam (85.8 Ci/mmol, Dupont Canada), isotopically diluted five-fold to a specific activity of 17.2 Ci/mmol, was added to a final concentration of 10 nM. The mixture was incubated for 45 min in the dark, on ice, with constant shaking, before irradiating with long wavelength ultraviolet light for 45 min using a Spectroline ENF 260C lamp at a distance of 6 cm. The membranes were subjected to repeated cycles of centrifugation (150,000*g*, 45 min) and resuspension until the radioactivity in the supernatant fell to close to the background, at which point the membranes were finally resuspended at a concentration of 15 mg/ml in the above buffer.

Solubilization of [^{3}H]Flunitrazepam Photoaffinity Labelled Membranes and Protein Precipitation

Photoaffinity labelled membranes were stirred on ice at 4°C and an equal volume of solubilization buffer containing 20 mM Tris-citrate pH 7.5, 0.5 M KCl, 3% 3-[-(cholimidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma Chemicals), 0.3% asolectin (Fluka) and protease inhibitors as noted above was added dropwise. After being stirred for 60 min at 4°C, the mixture was centrifuged for 75 min at 100,000*g*. To the supernatant containing the photoaffinity labelled receptor, a volume of trichloroacetic acid was added to a final concentration of 12% (w/v). Following incubation on ice for 15 min, the solution was centrifuged at 10,000*g* for 15 min and the protein pellets were washed twice with acetone.

Cyanogen Bromide Cleavage of the [^{3}H]Flunitrazepam Photoaffinity Labelled Receptor

Cyanogen bromide cleavage (Gross, 1967) of the photolabelled receptor was carried out by dissolving the solubilized and precipitated protein to a concentration of 10 mg/ml in 70% formic acid and adding a volume of 25% CNBr (Eastman Kodak) in formic acid solution to achieve a final amount of CNBr equal to that of the total protein (mg per mg basis). Following 24 hr incubation at room temperature in the dark, the reaction mixture was diluted into 15 volumes of distilled water, freeze dried twice and resuspended to about 10 mg protein per ml in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% NaN_3 buffer for subsequent immunopurification.

Immunoprecipitation of [^{3}H]Flunitrazepam Photoaffinity Labelled CNBr Peptides

Approximately 1.25 μl of neat sheep-raised anti-flunitrazepam polyclonal serum (Biodesign International) was added per pmol of the [^{3}H]flunitrazepam photoaffinity labelled CNBr peptides and the mixture was incubated for 2 hrs at 37°C. The antiserum titre was 1:7,680 (final dilution) as determined by the supplier with fluoroimmunoassays. The quantity of photolabelled peptide is expressed as pmol of radioligand. About 50 μl of 50% Protein G linked Sepharose 4B fast flow (Sigma Chemicals) in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% NaN_3 buffer was added per pmol of labelled peptide and the slurry was incubated for 24 hr at 4°C with constant mixing. The Protein G matrix was extensively washed by repeated cycles of centrifugation (10,000*g*, 15 min) and resuspension in a series of different solutions; (1) 10 mM Tris-HCl pH 7.4, 150 mM NaCl buffer, (2) 10 mM Tris-HCl pH 7.4 buffer, (3) 20% (v/v) ethanol, and (4) distilled

water. To specifically recover the labelled peptides, while avoiding spurious contaminants that were present in the precipitation assay, a large excess of free unlabelled flunitrazepam (Sigma Chemicals) was used to elute the immunoprecipitated peptides from the antibody-Protein G complex. A volume of flunitrazepam dissolved in 10% ethanol was added to the Protein G beads equivalent to 100 nmol free drug per pmol of labelled peptide. The slurry was incubated for 24 hr at 4°C with constant mixing, before the beads were pelleted by centrifugation and the supernatant containing the immunopurified peptides was removed and freeze-dried. The dried product was resuspended in distilled water and the insoluble free flunitrazepam was removed by ether extraction. After gentle mixing with diethyl ether/distilled water (3:1) to solubilize the drug, the ether phase was discarded and the aqueous phase containing the immunopurified peptide components was again freeze-dried. The [^{3}H]flunitrazepam photoaffinity labelled CNBr peptides were finally resuspended in 0.1% trifluoroacetic acid and stored at 4°C for analysis by HPLC.

Reversed-Phase HPLC of the Immunoprecipitated [^{3}H]Flunitrazepam Photoaffinity Labelled CNBr Peptides

Further purification of the immunoprecipitated peptides was achieved by reversed-phase HPLC using a Vydac C18, 25 cm x 0.46 cm column. The peptide components were eluted from the column using a linear gradient established with aqueous 0.05% trifluoroacetic acid (A) and acetonitrile/0.05% trifluoroacetic acid (B) at a flow rate of 1 ml per min. After 45 min of 1% B per min, the mixture was ramped to 90% B and

held for 5 min, then returned to 100% A. Fractions of 1 ml were collected and the [^{3}H]-radioactivity profile of the fractions was determined. Distinct peaks were pooled and concentrated by vacuum centrifugation for further study.

Deglycosylation of [^{3}H]Flunitrazepam Photoaffinity Labelled CNBr Peptides with N-Glycanase

The hydrolysis of all common classes of Asn-linked oligosaccharides at the ß-aspartyl-glycosylamine bond between the innermost GlcNAc and the asparagine residue is catalyzed by the enzyme, Peptide-N^4-(N-acetyl-ß-glucosaminyl)asparagine amidase (Genzyme Corporation), abbreviated *N*-Glycanase (Elder and Alexander, 1982; Tarentino et al., 1985). For deglycosylation experiments, pooled HPLC fractions were dried and resuspended in 0.5 M sodium phosphate pH 8.0, 0.5% SDS, and 50 mM ß-mercapto-ethanol and boiled for 5 min. Prior to the addition of 0.3 units of *N*-Glycanase enzyme, the sample was diluted three-fold and Nonidet P-40 (1.25% final) and 1,10 *ortho*-phenanthroline (10 mM final) were added. The mixtures were incubated for 18 h at 37°C, before the deglycosylated peptides were prepared for resolution by HPLC or SDS-PAGE.

Gel Electrophoresis of [^{3}H]Flunitrazepam Photoaffinity Labelled CNBr Peptides and Scintillation Counting

The immunoprecipitated photoaffinity labelled CNBr peptides and the components purified by reversed-phase HPLC were analyzed by Tricine SDS-PAGE for superior

resolution of species with molecular mass in the 1 to 100 kDa range (Schägger and von Jagow, 1987). Electrophoretic separations were carried out on gels which consisted of a 16.5%T, 6%C resolving slab, a 10%T, 3%C spacer gel and a 4%T, 3%C stacking gel (where T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide) and C denotes the percentage concentration of the crosslinker relative to the total concentration T). Immediately following electrophoresis, sample lanes were cut into 0.25 cm slices, eluted in 0.5 ml 2% SDS at 50°C overnight and then the radioactivity was determined by scintillation counting. Molecular weight (M_r) values of peptides were estimated by the migration profiles of parallel protein standards using low molecular weight rainbow-colored standards (Amersham).

Amino Acid Sequence Analysis

Amino-terminal sequence analysis was performed with an Applied Biosystems model 473A pulsed liquid/gas phase protein sequencer. Pooled HPLC samples were concentrated by vacuum centrifugation and stored at 4°C. Aliquots from the fractions were immobilized on polybrene-treated filters for sequencing by standard Edman degradation. The PTH-amino acids generated by each sequencer cycle were directly transferred to a fraction collector, instead of analyzing the derivatives by HPLC, and the amount of [^{3}H] in each cycle was assessed by scintillation counting. In some instances, the sequencer was allowed to operate with PITC omitted from the standard reaction protocol. This allowed estimation of the amount of non-specific elution of radioactivity during automated sequencing of particular samples.

RESULTS

Photoaffinity Labelling of the $GABA_A$ Receptor with [^{3}H]Flunitrazepam

To characterize the site of photoincorporation by [^{3}H]flunitrazepam on the $GABA_A$ receptor, a photoaffinity labelled peptide component from bovine cerebral cortex was purified by immunoprecipitation with a polyclonal antiserum raised against free flunitrazepam and with the precipitating reagent, Protein G-linked Sepharose. In addition to quantitatively precipitating free [^{3}H]flunitrazepam, the anti-flunitrazepam serum was shown to immunoprecipitate photoaffinity labelled peptides by specifically recognizing the [^{3}H]flunitrazepam ligand covalently associated with the $GABA_A$ receptor (see below). After confirming (by SDS-PAGE) that the photoincorporation of [^{3}H]flunitrazepam with the $GABA_A$ receptor from bovine cerebral cortex was associated with a major 53 kDa protein (Fig. 2.1), previously defined as the α subunit(s) (Fuchs et al., 1988; Stephenson et al., 1990) and that there was no significant labelling of other species, the photoaffinity labelled protein was solubilized and subjected to specific chemical cleavage at Met residues by treatment with CNBr.

Immunoprecipitation of [^{3}H]Flunitrazepam Photolabelled Peptides

The concentration-dependence of immunoprecipitation of [^{3}H]flunitrazepam photoaffinity labelled CNBr peptides with anti-flunitrazepam serum was investigated to assess the optimal antibody concentration for use in large scale purification (Fig. 2.2). A maximum immunoprecipitation of 70% $\pm$ 9% was achieved with less than 1 μl of neat

antiserum per pmol of [^{3}H]flunitrazepam photolabelled CNBr peptide. Non-specific adsorption of labelled peptides to the Protein G matrix was measured in the absence of antiserum and represented less than 1% of the total yield.

Batch immunoprecipitation was used to purify [^{3}H]flunitrazepam photoaffinity labelled CNBr peptides in quantities sufficient for further characterization. About 50% of the total yield of immunoprecipitated product could be specifically eluted from the antibody - Protein G complex by incubation with free flunitrazepam. The radioactivity profile of the immunopurified peptides resolved by Tricine SDS-PAGE (Fig. 2.3A) demonstrated that CNBr cleavage of the $GABA_A$ receptor preparation generated a [^{3}H]flunitrazepam photoaffinity labelled peptide of 5.5 kDa molecular mass, with a minor component which resolved as a 2.5 kDa species. Unfortunately, the inadequate amount of purified protein precluded the use of silver stain for protein detection. The elution profile from reversed-phase HPLC of the immunoprecipitated peptides (Fig. 2.3B) displays three apparent peaks of radioactivity, arbitrarily marked (i), (ii) and (iii). While the [^{3}H]-peak marked (iii) invariably represented 55 to 60% of the total radioactivity loaded onto the column, the relative amount of radioactivity in the other two peaks varied between peptide preparations. The percentage of [^{3}H] that eluted with the peaks ranged from 17 to 35% for (i) and from 8 to 26% for (ii). However, the combined cpm in peaks (i) and (ii) routinely represented 40 to 45% of the total radioactivity.

Characterization of Radioactive Components resolved by Reversed-Phase HPLC of Immunopurified [^{3}H]Flunitrazepam Peptides

The pooled fractions from each of the radioactive HPLC elution peaks were resolved by Tricine SDS-PAGE for determination of the molecular mass of the [^{3}H]flunitrazepam labelled CNBr peptide components. In addition, the samples were treated with the deglycosylation enzyme, *N*-Glycanase, to assess whether the radiolabelled peptides contained asparagine-linked oligosaccharides. The radioactivity profile of HPLC peak (iii) resolved by SDS-PAGE shows that the [^{3}H]flunitrazepam label is associated with a major 5.4 kDa peptide (Fig. 2.4A), i.e. a profile similar to the unfractionated peptides (Fig. 2.3A). Following deglycosylation of peak (iii) with *N*-Glycanase, the apparent molecular mass of the peptide was reduced to 3.2 kDa (Fig. 2.4B). Reversed-phase HPLC of peak (iii) after digestion with *N*-Glycanase gave a small, but consistent, shift to a higher percentage of acetonitrile required for the elution of this peptide from the column. In both the SDS-PAGE and HPLC profiles, some undigested peak (iii) apparently remained after *N*-Glycanase treatment and this accounted for approximately 20% of the total radioactivity.

HPLC peak (ii) resolved by SDS-PAGE as a broad band of radioactivity with molecular mass ranging from 8.7 to 12.1 kDa. After digestion with *N*-Glycanase, peak (ii) resolved to a more distinct, but still broad, band of [^{3}H] that corresponds to a radiolabelled peptide of about 9 kDa. It was not possible to resolve the radioactivity present in HPLC peak (i) to a distinct protein band by electrophoresis. In repeated attempts, the radioactivity showed a diffuse migration pattern spanning the middle portion

of the gel which did not vary when the pore size of the gel was altered. The incubation of peak (i) with N-Glycanase had no effect on the apparent inability of this fraction to be resolved by gel electrophoresis.

Automated Amino Acid Sequencing of [^{3}H]Flunitrazepam Photolabelled Components

The [^{3}H]flunitrazepam photoaffinity labelled peptides purified by HPLC (see Fig. 2.3B), were subjected to direct automated sequencing to measure the release of radioactivity during each cycle. The pattern of release of radioactive PTH-amino acids generated by Edman degradation of the [^{3}H]flunitrazepam photolabelled peptides present in HPLC peak (iii) shows that a maximum release of [^{3}H] occurred in sequencer cycle #12 (Fig. 2.5). The release of radioactivity remained elevated for about four sequencer cycles before returning to baseline levels. The [^{3}H] elution seen in cycles #13 through #16 is most likely due to N-terminal cleavage from residual peptide containing the [^{3}H]flunitrazepam associated amino acid, originally residue #12, and/or incomplete extraction of the modified amino acid. It is probable that the covalent incorporation of the ligand with a particular amino acid adversely affects the efficiency of the Edman degradation reaction. The moderate level of radioactivity seen in the first cycle likely results from desorption of peptide from the filter-cartridge system of the sequencer.

The pattern of radioactive release from automated sequencing of HPLC peaks (i) and (ii) were indicative of non-specific elution during the Edman degradation cycles (Fig 2.6). Therefore, duplicate aliquots of these samples were processed in the same manner as for standard sequencing, except that PITC was omitted from the reaction chemistry.

The elution of [^{3}H] during the cycles from sham sequencing of peaks (i) and (ii) was parallel to that obtained for the standard Edman reaction cycles.

DISCUSSION

The identity of specific amino acid residue(s) in native $GABA_A$ receptors that are directly involved in benzodiazepine binding has remained an area of intense interest. One approach used extensively has been to specifically and irreversibly label the benzodiazepine binding site with the photoactivatable agonist, [^{3}H]flunitrazepam. While strong evidence has been reported to show that the major site of [^{3}H]flunitrazepam photoaffinity labelling occurs on the $GABA_A$ receptor α-subunit, the precise position of the photolabel on the polypeptide has not previously been established.

To identify the [^{3}H]flunitrazepam photoaffinity labelling site on the $GABA_A$ receptor, a purified preparation of labelled peptide was required in sufficient quantities to allow for characterization by conventional biochemical techniques. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labelled peptides from a crude brain preparation. The [^{3}H]flunitrazepam photoaffinity labelled peptides generated by CNBr cleavage were precipitated with an antiserum directed against free flunitrazepam, that also specifically recognized the covalently attached ligand. While the major component evident in the immunopurified preparation was a 5.5 kDa photolabelled peptide when resolved by SDS-PAGE, three distinct peaks of radioactivity were resolved by reversed-phase HPLC.

The [^{3}H]flunitrazepam present in HPLC peak (iii) represented the majority (approximately 60%) of the total immunoprecipitated radioactivity and was shown, by SDS-PAGE, to be associated with a peptide of apparent molecular mass 5.4 kDa. It was also shown that this photolabelled peptide contains asparagine-linked carbohydrate, since after *N*-Glycanase digestion, it migrated as a 3.2 kDa peptide. Recognizing that: (1) CNBr specifically cleaves peptide bonds on the carboxyl-side of Met residues with high efficiency, except for Met-Thr and Met-Ser bonds which are essentially resistant to CNBr and (2) that asparaginyl-linked glycosylation exclusively occurs at the consensus sequence of Asn-X-Thr or Asn-X-Ser, the origin of the photolabelled peptide can be mapped to the known amino acid sequence of the N-terminal domain of the $GABA_A$ receptor α_1-subunit (see Fig. 2.7). Considering the potential sites for cleavage by CNBr and for asparaginyl glycosylation, the only peptide that could be generated from the α-subunit to contain Asn-linked carbohydrate and to resolve by electrophoresis as described above is Ala91 to Met114. This peptide has a predicted molecular mass of 3.1 kDa, without consideration given for glycosylation, which is close to the estimates obtained by SDS-PAGE analysis. The other CNBr peptide that could be generated from the α-subunit to contain Asn-linked oligosaccharide is Gln1-Met59, which would have a molecular mass after deglycosylation of greater than 6.5 kDa. Since previous attempts to sequence the α-subunit indicated that the polypeptide was amino-terminal blocked (Schofield et al., 1987), the peptide beginning with Gln1 would also be refractory to the Edman chemistry. Furthermore, the peptide bonds carboxyl to Met90 and Met114 have previously been shown to be susceptible to CNBr cleavage, and sequencing of the peptides Ala91-Ala109

and Pro115-Thr130 provided the information necessary for the first cloning of the $GABA_A$ receptor subunits (Schofield et al, 1987). The [^{3}H]flunitrazepam photolabelled peptide has been mapped to the α_1 subunit, as this isoform has been shown to occur in the vast majority of native $GABA_A$ receptors from bovine cerebral cortex (Duggan and Stephenson, 1990). While other, less abundant, isoforms of the α-subunit have been identified in cortical $GABA_A$ receptors, the interpretation for the origin of the photolabelled peptide is not compromised, since the isoforms possess a high degree of sequence homology throughout this domain.

The pattern of release of radioactive PTH-amino acids obtained from automated Edman degradation of the major photolabelled peptide (Ala91-Met114) indicated the [^{3}H]flunitrazepam is covalently associated with the twelfth residue, which corresponds to His in position 102 of the α_1 subunit. The photoincorporation of [^{3}H]flunitrazepam with His102 is consistent with the findings of hydroxylamine cleavage experiments that demonstrated photolabelling occurred prior to Asn103, as well as previous reports that predicted the site occurred within limited subunit domains (see Chapter 3). The involvement of a histidine residue in the interaction of benzodiazepines with the $GABA_A$ receptor was implicated in earlier studies that investigated the effects of chemical modification and the pH dependency of radioligand binding (Lambolez and Rossier, 1987; Lambolez et al., 1989). Subsequently, His102 of the α_1 subunit was shown, by point mutation, to be required for the high affinity binding of benzodiazepine agonists in recombinantly expressed $GABA_A$ receptors (Wieland et al, 1992). In addition, the evidence presented here corroborates the preliminary microsequencing data presented by

Olsen and Smith (1994) which implicated α-subunit His102 as the site for photo-incorporation by [^{3}H]flunitrazepam.

The chemical nature of the radioactivity present in HPLC peaks (i) and (ii) has not been established. HPLC peak (ii), which accounted for 10-25% of the total radioactivity, resolved by SDS-PAGE to a molecular mass ranging from 9 to 12 kDa, with deglycosylation causing a marginal shift in the gel profile that indicated a 9 kDa photolabelled peptide. The radioactivity profile obtained from direct sequencing of peak (ii) did not suggest the [^{3}H]flunitrazepam label was associated with a particular amino acid residue. Automated sequencing in the absence of PITC demonstrated that the release of radioactivity seen during the first few cycles of Edman degradation was not due to the generation of radiolabelled PTH-amino acids resulting from N-terminal cleavage of a labelled peptide. While a reliable prediction for the origin of the photolabelled peptide is difficult, we can speculate that a 9 kDa peptide containing [^{3}H]flunitrazepam labelled His102 could result from incomplete cleavage at one or more of the potential CNBr sites in the N-terminal domain of the α-subunits. Alternatively, the peptide may have been generated from a different domain of the α-subunit or from another subunit subtype, thereby representing a minor fraction of photolabel incorporated with a residue other than His102. Recent studies have suggested the benzodiazepine binding domain is made up of determinants from both the α and γ subunits. Site-directed mutagenesis has found that Thr142 of the γ-subunit is involved in conferring the modulatory effects of benzodiazepine ligands (Mihic et al., 1995) and previous photoaffinity labelling experiments have shown data that suggest some [^{3}H]flunitrazepam

label may incorporate with the γ-subunit (Stephenson and Duggan, 1989). The apparent inability to resolve the radioactivity present in HPLC peak (i) by electrophoresis suggested the [^{3}H]flunitrazepam was not associated with a distinct peptide. In addition, the radioactivity profiles obtained from automated sequencing of peak (i) were not consistent with the release of labelled PTH-amino acids from the Edman degradation reaction. It is possible that the peak (i) fraction of the immunopurified product may represent free [^{3}H]flunitrazepam or ligand incorporated with carbohydrate or some other non-protein molecule.

In conclusion, evidence has been presented that demonstrates photoaffinity labelling of the $GABA_A$ receptor with [^{3}H]flunitrazepam leads to the covalent association of the ligand with His102 of the α-subunit. Despite intentions to characterize the photolabelled peptide components by microsequence analysis, current technical limitations and insufficient yields of the purified peptides have precluded the identification of the PTH amino acids generated during each cycle. Therefore, while His102 of the α-subunit is likely to be the major site of photoincorporation, it is not possible to substantiate the possible existence of other amino acids that may be photolabelled by [^{3}H]flunitrazepam.

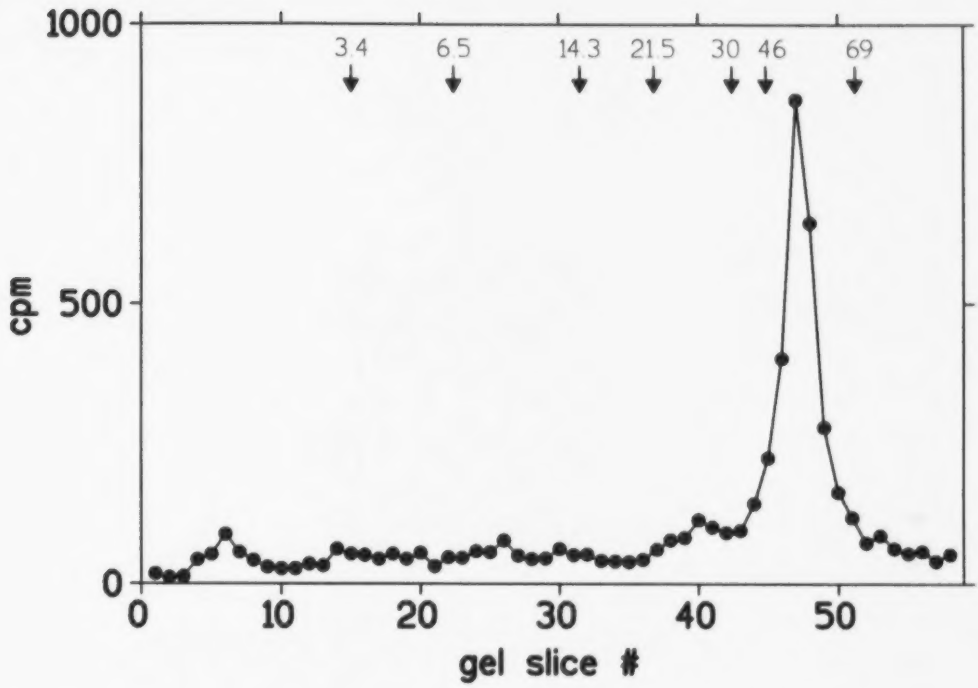


Figure 2.1. $GABA_A$ receptors from bovine cerebral cortex photoaffinity labelled by [^{3}H]flunitrazepam. The radioactivity profile of [^{3}H]flunitrazepam photolabelled $GABA_A$ receptors resolved by Tricine SDS-PAGE is shown. Determination of the cpm per gel slice was performed as described in "Material and Methods" section. The numerals above each arrow indicate the relative position of the molecular mass standards (expressed in kDa). The labelled receptor resolves to a single peak of radioactivity that corresponds to a protein band with an apparent molecular mass of 53 kDa. The profile shown is representative of numerous gels ($n > 10$) from distinct photolabelled membrane preparations.

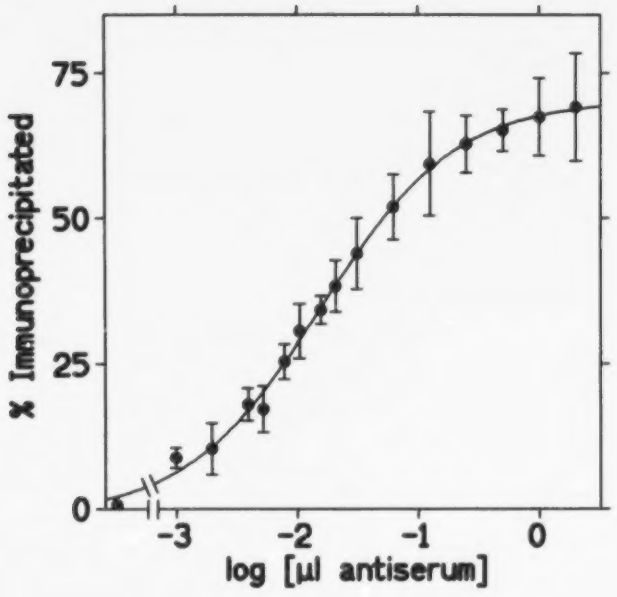


Figure 2.2. Concentration-dependent immunoprecipitation of [^{3}H]flunitrazepam photoaffinity labelled CNBr peptides by anti-flunitrazepam polyclonal serum. The preparation of [^{3}H]flunitrazepam photoaffinity labelled peptides and the immunoprecipitation of these peptides with anti-flunitrazepam serum and Protein G matrix was carried out as described under "Materials and Methods". The concentration-dependency of immunoprecipitation was investigated by incubating 0.5 pmol of photolabelled peptides (20 μl) with different amounts of polyclonal antiserum diluted into 200 μl buffer and 50 μl of 50% Protein G matrix. After extensive washing, the Protein G beads were pelleted by centrifugation and the radioactivity was determined by direct scintillation counting. The percentage immunoprecipitated was calculated from the amount of [^{3}H] associated with the Protein G matrix compared to the total amount of labelled peptide added to the sample. The data are expressed as the mean $\pm$ S.D. of three separate assays and the fit is to a simple binding model.

Figure 2.3. Immunoprecipitated [^{3}H]flunitrazepam photoaffinity labelled CNBr peptides resolved by SDS-PAGE and HPLC. (A) Representative radioactivity profile of immunopurified [^{3}H]flunitrazepam photolabelled peptides resolved by Tricine SDS-PAGE is shown as previously described in the legend to Fig. 2.1. The major peak of radioactivity corresponds to a peptide band with approximate molecular mass of 5.5 kDa, while the smaller component resolves as a 2.5 kDa peptide. Data shown are typical of several preparations of immunopurified peptides. (B) Immunoprecipitated [^{3}H]flunitrazepam photolabelled peptides were resolved by reversed-phase HPLC as described (see Methods section). Data are shown as the representative radioactivity profile of the elution of [^{3}H] in 1 ml fractions as determined by scintillation counting of 100 μl aliquots. Approximately 14,000 cpm of the immunoprecipitated peptides were loaded directly onto the column and the recovery of [^{3}H] was greater than 93%. The peak marked as (i) eluting at fraction #21 contained 4700 cpm, the small peak marked (ii) eluting at fraction #26 contained 1100 cpm and the largest peak (iii) eluting at fraction #33 contained approximately 7,250 cpm.

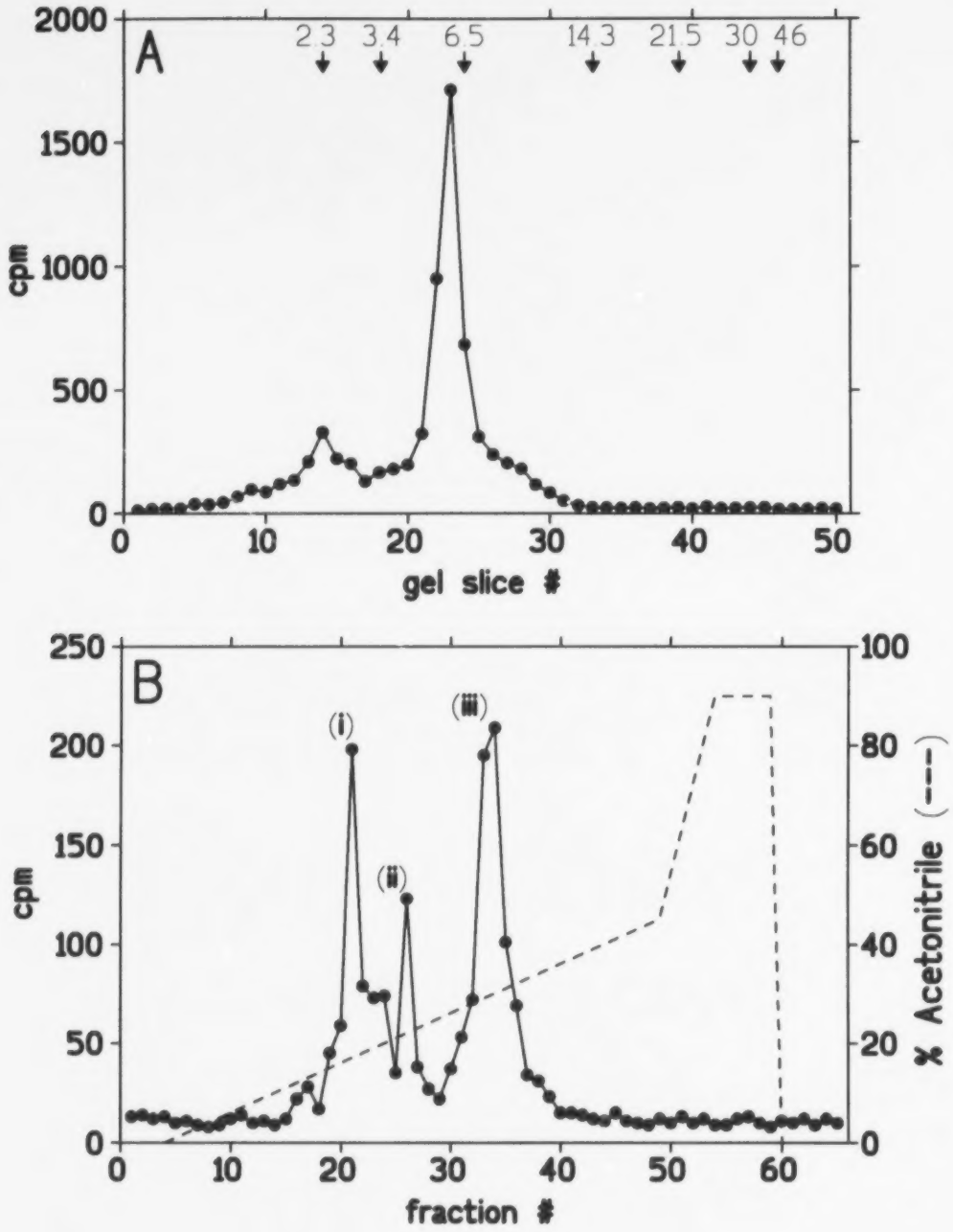
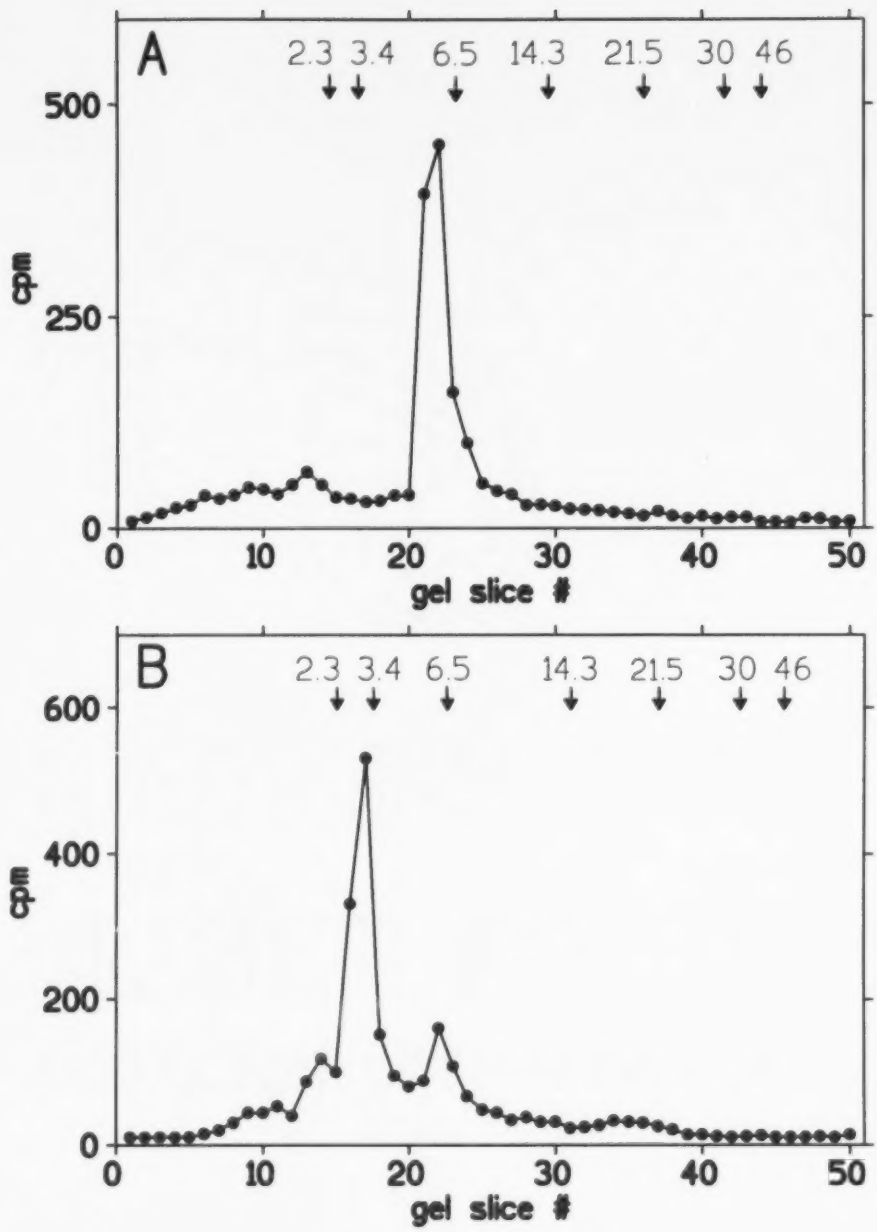

A
2.3
3.4
6.5
14.3
21.5
30
46
cpm
gel slice #
B
(i)
(ii)
(iii)
cpm
fraction #
% Acetonitrile (---)

Figure 2.4. Peak (iii) from reversed-phase HPLC of immunopurified [^{3}H]flunitrazepam CNBr peptides, resolved by SDS-PAGE before and after treatment with *N*-Glycanase. The [^{3}H]-profiles of native and deglycosylated peptides of HPLC peak (iii) resolved by SDS-PAGE are shown as described in the legend to Fig. 2.1. (A) Peak (iii) from reversed-phase HPLC of immunoprecipitated peptides resolves by SDS-PAGE to an apparently single radiolabelled peptide with molecular mass of about 5.4 kDa. (B) Following treatment of the peptides eluted in peak (iii) with *N*-Glycanase, as described under "Materials and Methods", the major radiolabelled species resolves to a 3.2 kDa peptide, with some residual 5.4 kDa peptide evident.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500
250
0
cpm
0 10 20 30 40 50
gel slice #
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200
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cpm
0 10 20 30 40 50
gel slice #

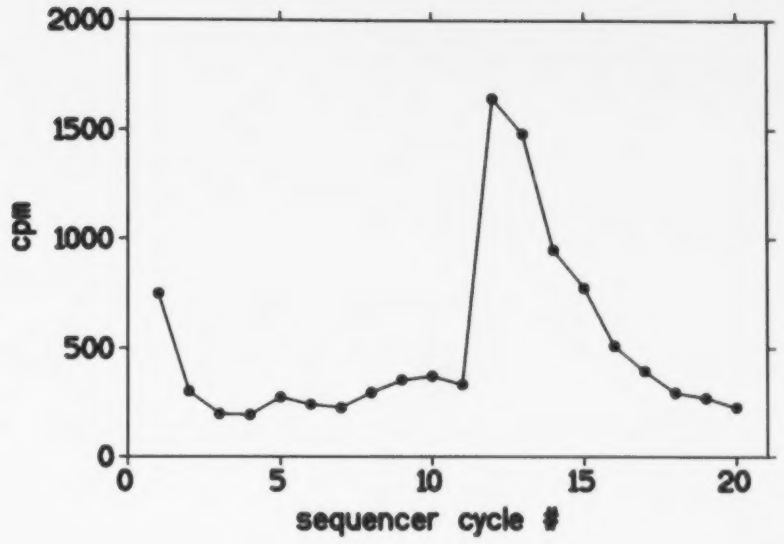
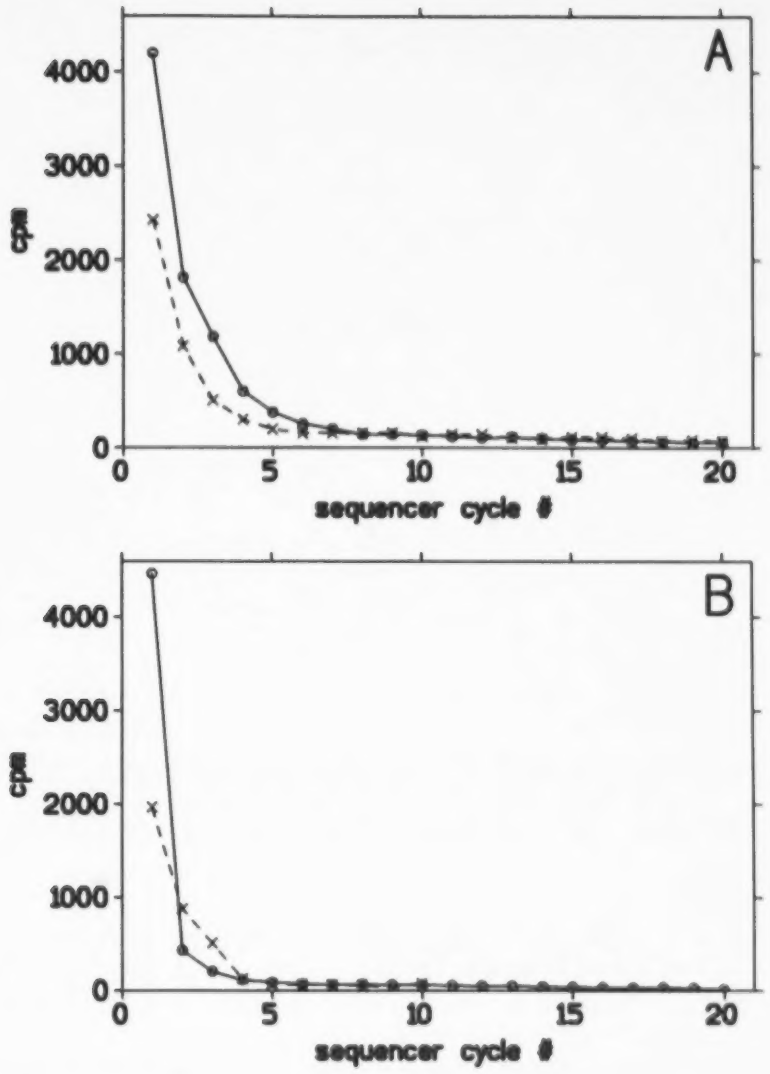


Figure 2.5. Release of radioactive amino acids during automated Edman degradation of [^{3}H]Flunitrazepam labelled CNBr peptides from HPLC Peak (iii). The amount of [^{3}H] associated with the PTH-amino acids generated by each cycle of Edman degradation of the photolabelled peptides present in HPLC peak (iii) was determined as described (see Methods section). The radioactivity shown is that observed and has not been corrected for repetitive yield of sequencer cycles. Approximately 30% of the radioactivity loaded onto the sequencer was recovered in the fractions collected from each cycle, about 25% remained on the filter and cartridge seal, and the remainder was presumably lost in the washes.

Figure 2.6. Release of radioactivity from HPLC peaks (i) and (ii) subjected to automated Edman degradation. The amount of [^{3}H] that eluted with each cycle during standard conditions of Edman degradation and when PITC was omitted from the automated reaction was investigated (see Methods section). Equivalent amounts of radioactivity were loaded onto the sequencer for all instances. (A) Automated sequencing of HPLC peak (i) in the presence (○) and absence (x) of PITC. Of the [^{3}H] recovered, 28% was released during the cycles of Edman degradation, 4% was left on the filter and cartridge seal, and 68% was presumed lost in washes. (B) Automated sequencing of HPLC peak (ii) in the presence (○) and absence (x) of PITC; the recovery of the radioactivity loaded onto the sequencer was 17% in the normal reaction cycles, 3% remained on the filter and 80% was lost in washes. The radioactivity released from peaks (i) and (ii) during the sham sequencing cycles (minus PITC) was slightly reduced, while the amount of [^{3}H] presumed to be lost in washes was increased, compared to that for the standard sequencer runs.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3000
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cpm
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15
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sequencer cycle #
B
4000
3000
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1000
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cpm
0
5
10
15
20
sequencer cycle #

10
Gln Pro Ser Leu Gln Asp Glu Leu Lys Asp Asn Thr Thr Val Phe Thr Arg Ile Leu Asp
30
Arg Leu Leu Asp Gly Tyr Asp Asn Arg Leu Arg Pro Gly Leu Gly Glu Arg Val Thr Glu
50
Val Lys Thr Asp Ile Phe Val Thr Ser Phe Gly Pro Val Ser Asp His Asp Met Glu Tyr
70
Thr Ile Asp Val Phe Phe Arg Gln Ser Trp Lys Asp Glu Arg Leu Lys Phe Lys Gly Pro
90
Met Thr Val Leu Arg Leu Asn Asn Leu Met Ala Ser Lys Ile Trp Thr Pro Asp Thr Phe
110
Phe His Asn Gly Lys Lys Ser Val Ala His Asn Met Thr Met Pro Asn Lys Leu Leu Arg
130
Ile Thr Glu Asp Gly Thr Leu Leu Tyr Thr Met Arg Leu Thr Val Arg Ala Glu Cys Pro
150
Met His Leu Glu Asp Phe Pro Met Asp Ala His Ala Cys Pro Leu Lys Phe Gly Ser Tyr
170
Ala Tyr Thr Arg Ala Glu Val Val Tyr Glu Trp Thr Arg Glu Pro Ala Arg Ser Val Val
190
Val Ala Glu Asp Gly Ser Arg Leu Asn Gln Tyr Asp Leu Leu Gly Gln Thr Val Asp Ser
210
Gly Ile Val Gln Ser Ser Thr Gly Glu Tyr Val Val Met Thr Thr His Phe His Leu Lys
Arg Lys Ile Gly Tyr Phe Val Ile Gln Thr Tyr Leu Pro Cys Ile Met Thr Val .....

Figure 2.7. Partial Amino Acid Sequence of the Bovine $GABA_A$ Receptor α_1 Subunit. The $GABA_A$ receptor sequence of the large extracellular amino-terminal domain of the α_1 subunit, from Gln1 to the first putative transmembrane domain, is shown. Met residues are in bold print with the potential cleavage sites for CNBr indicated by ↓, the potential sites for N-linked glycosylation are underlined (Asn X Thr), the large bracket between Asn103-Gly104 marks the only potential site in the subunit for specific cleavage by hydroxylamine and the putative membrane spanning region is marked by the shaded box above the initial residue Phe226.

CHAPTER 3

Mapping of $GABA_A$ Receptor Sites that are Photoaffinity Labelled by [^{3}H]Flunitrazepam and [^{3}H]Ro15-4513

A version of this chapter has been accepted for publication. Duncalfe, L.L. and S.M.J. Dunn, 1995, Eur. J. Pharmacol. Mol. Pharmacol, in press.

INTRODUCTION

The benzodiazepine ligands which interact with the $GABA_A$ receptor can be grouped into three categories; agonists, inverse agonists and antagonists. These benzodiazepines are allosteric modulators of $GABA_A$ receptors, in that the classical agonists like flunitrazepam potentiate, while the inverse agonists reduce, GABA-mediated chloride conductances. Ro15-4513 is a partial inverse agonist that binds competitively to sites that are sensitive to classical agonists and antagonists, as well as to a sub-population of diazepam-insensitive $GABA_A$ receptors that are found mainly in the cerebellum (<20% of sites) and to a minor extent (<2.5% of sites) in the cerebral cortex (Sieghart et al., 1987b; Turner et al., 1991).

In the present study, [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 photoaffinity labelled $GABA_A$ receptor preparations from the cerebral cortex and cerebellum have been subjected to specific chemical cleavage with hydroxylamine. Hydroxylamine cleaves specifically at an Asn-Gly bond, a peptide bond that occurs with a probability of the order of 1/400 for most proteins (Bornstein and Balian, 1977). The resulting labelled peptides were analyzed by SDS-PAGE for molecular mass determination and mapped to the known amino acid sequence of bovine $GABA_A$ receptor polypeptides. With respect to the subunit isoforms for which the bovine sequence has not been determined (i.e., α_5 and α_6), the rat and human sequences were used. The hydroxylamine cleavage patterns demonstrate differences in the sites of photoaffinity labelling by the classical agonist, [^{3}H]flunitrazepam, and partial inverse agonist, [^{3}H]Ro15-4513.

MATERIALS AND METHODS

Preparation and Photoaffinity Labelling of Bovine Brain Membranes

Brain membranes were prepared from bovine cerebral cortex or cerebellum as previously described (Chapter 2) and resuspended in 20 mM Tris-citrate pH 7.5 containing 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml bacitracin, 0.02% NaN_3 to a final protein concentration of 1 mg/ml, determined by the Bradford method (1976). [^{3}H]Flunitrazepam (5 nM final) or 10 nM [^{3}H]Ro15-4513 (Dupont Canada, 85.8 and 27.4 Ci/mmol, respectively) was added to the brain membranes and the mixture was incubated on ice for 45 min prior to ultraviolet irradiation (Spectroline ENF 260C lamp, long wavelength) at a distance of 6 cm for 45 min on ice with constant stirring. Non-specific photoaffinity labelling was determined in the presence of excess unlabelled ligand. For [^{3}H]Ro15-4513 photoaffinity labelling of the cerebellar diazepam-insensitive population of $GABA_A$ receptor sites, 5 μM unlabelled flunitrazepam (Sigma Chemicals) was included in the incubation mixture. After photoaffinity labelling, the preparations were subjected to repeated cycles of centrifugation (150,000*g*, 45 min) and resuspension to remove free ligand. The labelled membranes were resuspended at a final concentration of 15 mg/ml in the above buffer.

Solubilization of Photoaffinity Labelled Membranes and Protein Precipitation

An equal volume of solubilization buffer; 20 mM Tris-citrate pH 7.5, 0.5 M KCl, 3% CHAPS (Sigma Chemicals), 0.3% asolectin (Fluka) and protease inhibitors as noted above was added dropwize to the photoaffinity labelled membranes followed by stirring on ice for 60 min prior to centrifugation at 100,000*g* for 60 min. The supernatant containing the solubilized protein was added to an equal volume of 24% (w/v) trichloroacetic acid, incubated on ice for 15 min and centrifuged (10,000*g*, 15 min). The protein pellets were washed twice with acetone before resuspension in the solution for the hydroxylamine cleavage reaction.

Hydroxylamine Cleavage of [^{3}H]Flunitrazepam and [^{3}H]Ro15-4513 Photoaffinity Labelled Receptor Preparations

Hydroxylamine cleavage of the labelled receptor was performed as described (Bornstein and Balian, 1977). The labelled protein (3 to 5 mg/ml final) was added to a solution of 2 M hydroxylamine, 6 M guanidine, 4.5 M lithium hydroxide (Fisher Scientific) and incubated for 4 hrs at 45°C, maintaining the pH at 9 with lithium hydroxide as titrant. The reaction was terminated by acidification to pH less than 3 using concentrated formic acid. The mixture was desalted on a Sephadex G-25 superfine column (1.5 x 25 cm) in 9% formic acid. The fractions containing radioactivity that eluted in the void volume were pooled, freeze-dried and resuspended in buffer for subsequent resolution by SDS-PAGE. The hydroxylamine cleavage reaction was repeated at least twice for all of the photoaffinity labelled receptor preparations studied.

SDS-PAGE of Hydroxylamine Generated Peptides and Scintillation Counting

The photoaffinity labelled receptor preparations and peptides generated from chemical cleavage by hydroxylamine were analyzed by SDS-PAGE under reducing conditions using a Tricine buffer system for superior resolution (Schägger and von Jagow, 1987). The gels utilized for electrophoretic separations consisted of a resolving slab of 10%T, 3%C, a spacer gel of 7%T, 3%C and a stacking gel of 4%T, 3%C; where %T = (gram acrylamide + bis-acrylamide) per 100 ml and %C = gram bis-acrylamide per (gram acrylamide + bis-acrylamide). Immediately following electrophoresis, sample lanes were cut into 0.25 cm slices, eluted in 0.5 ml 2% SDS at 50°C overnight and radioactivity was determined by scintillation counting after addition of 5 ml Ecolite scintillant (Fisher Scientific). Molecular weight values of peptides were estimated by the migration profiles of standard proteins electrophoresed in parallel lanes on each gel, using both high and low molecular weight rainbow-colored standards (Amersham Canada). The photoaffinity labelled receptor preparations and hydroxylamine generated peptides were resolved by electrophoresis on two to three separate gels for molecular weight estimations.

pH Dependency of Radioligand Binding

Brain membranes prepared from bovine cerebral cortex were diluted to a final protein concentration of 0.4 mg/ml in 50 mM Tris, 25 mM acetic acid, 25 mM 2-[N-morpholino]-ethanesulfonic acid (MES), 100 mM NaCl, 0.02% NaN_3 buffer titrated with either 1 N HCl or 1 N NaOH to pH values ranging from 5 to 9. Over this pH range,

the ionic strength of this buffer mixture remains essentially constant (Ellis and Morrison, 1982). [^{3}H]Flunitrazepam or [^{3}H]Ro15-4513 (15 nM final) was added to the membranes prior to incubation for 60 min at 4°C. Non-specific binding was determined in the presence of 10 μM cold ligand added to parallel sample sets. Duplicate aliquots of each sample were filtered under vacuum through Whatman GF/C filters using a Hoefer filtration apparatus, and the filters were immediately washed with two 5-ml volumes of ice cold pH 7 buffer. The filters were dried, 5 ml of scintillation fluid added and samples were counted for [^{3}H].

RESULTS

Photoaffinity Labelled Receptor Preparations from Bovine Cerebral Cortex

Photoaffinity labelling of bovine brain membranes from cerebral cortex with [^{3}H]flunitrazepam led to covalent incorporation of the ligand which, by SDS-PAGE, was shown to be associated with a major protein band of 53 kDa (Fig. 3.1A), corresponding to $GABA_A$ receptor(s) α subunits (Fuchs et al., 1988). The major α-subunit isoform found in cortical $GABA_A$ receptors is the α_1 subunit, although other less abundant isoforms have been identified (Duggan and Stephenson, 1990). As shown in Fig. 3.1B, SDS-PAGE analysis of the hydroxylamine cleavage pattern of the [^{3}H]flunitrazepam labelled receptor preparation indicated that a major radiolabelled peptide of approximate mass of 12 kDa is generated. Since no residual 53 kDa radiolabelled species from the receptor preparation is evident following hydroxylamine treatment, the extent of specific

chemical cleavage is presumed to be complete. The primary amino acid sequences of the α_1, α_2 and α_5 subunits are known to possess only one asparagine-glycine bond, located at residues 103/104 (α1 subunit numbering), that is susceptible to specific cleavage by hydroxylamine. Cleavage of the subunit at this peptide bond would generate only two major products differing greatly in size; 1 - 103 and 104 - 430 of approximate 10 kDa and 43 kDa molecular mass, respectively. The α_3, α_4 and α_6 subunits also contain the Asn-Gly bond between residues 103/104, plus an additional hydroxylamine cleavage site located 21 residues carboxy-terminal to the first site. The ß, γ and δ subunits of the $GABA_A$ receptor do not contain an Asn - Gly peptide bond, so hydroxylamine treatment will not specifically cleave these subunits. Therefore, the 12 kDa peptide generated from hydroxylamine cleavage of [^{3}H]flunitrazepam photoaffinity labelled receptors demonstrates that the major site of labelling lies prior to Asn residue 103 of the $GABA_A$ receptor α subunit(s). The minor 6.5 kDa peptide component evident in the radioactivity profiles of the [^{3}H]flunitrazepam labelled preparations (fig. 3.1A, 3.1B) was variable and is presumed to represent a non-specific degradative product.

[^{3}H]Ro15-4513 photoaffinity labelling of bovine cerebral cortical membranes generated a covalently labelled 55 kDa protein and a secondary component of 65 kDa, as resolved by gel electrophoresis (fig 3.1C). As seen in Fig. 1D, hydroxylamine treatment of the [^{3}H]Ro15-4513 photoaffinity labelled receptor preparation cleaved both radiolabelled components to generate a peptide with an apparent mass of 40 kDa. These results indicate that both the 55 and 65 kDa photolabelled polypeptides from the receptor preparation correspond to α subunit(s) and that the doublet peak (fig. 3.1C) likely

represents labelling of multiple α subunit isoforms. Further, the hydroxylamine cleavage pattern demonstrates that the major site of [^{3}H]Ro15-4513 labelling occurs on the α subunit(s) between the Gly residue at position 104 and the carboxy-terminus. The higher molecular weight species observed at the top of the gel in Fig. 3.1D is due to the poor solubility of the hydroxylamine cleaved peptides which results in some precipitation at the spacer - resolving gel interface (see below).

Photoaffinity Labelled Receptor Preparations from Bovine Cerebellum

$GABA_A$ receptors located in the cerebellum can be classified into two distinct populations; diazepam-sensitive and diazepam-insensitive. The diazepam-insensitive binding sites correlate with the presence of an α_6 subunit in the $GABA_A$ receptor (Lüddens et al., 1990). In Fig. 3.2A, the total population of cerebellar bindings sites for [^{3}H]Ro15-4513 have been photoaffinity labelled and resolved by SDS-PAGE to a 58 kDa protein band. The peptides resulting from hydroxylamine treatment of this labelled receptor preparation (Fig. 3.2B) resolve to a major 43 kDa component. These results demonstrate that, as in cortex, the site of [^{3}H]Ro15-4513 photoaffinity labelling occurs between residue Gly 104 and the carboxy-terminus (α_1 subunit numbering) in the cerebellar population of diazepam-sensitive $GABA_A$ receptors. Photoaffinity labelling of cerebellar preparations with [^{3}H]Ro15-4513 in the presence of excess unlabelled flunitrazepam will lead to selective incorporation of the label into the diazepam-insensitive receptor population. This covalently labelled protein resolves as a 57 kDa species by SDS-PAGE (Fig. 3.2C) and the peptides generated by hydroxylamine cleavage

of this receptor preparation resolve to 14 kDa (Fig. 3.2D). Therefore, the [^{3}H]Ro15-4513 photoaffinity labelling site for the diazepam-insensitive receptors occurs within residues 1 - 101 (α_6 subunit numbering). Since the diazepam-insensitive population of [^{3}H]Ro15-4513 binding sites comprise less than 20% of the $GABA_A$ receptors in the cerebellum, the 14 kDa hydroxylamine peptides resulting from these receptors are not discernible in the preparation generated from the total cerebellar sites due to the background noise present in the radioactivity profile (Fig. 3.2B).

[^{3}H]Flunitrazepam photoaffinity labelling to bovine cerebellar $GABA_A$ receptors and hydroxylamine peptides generated from the labelled receptor preparations parallels the cleavage patterns obtained for the receptor population of the cerebral cortex, i.e., the covalently attached [^{3}H]flunitrazepam label was shown to be associated with a major protein band of 55 kDa (Fig. 3.3A) and the peptides resulting from hydroxylamine treatment resolved by SDS-PAGE as a somewhat broad band with the major peak of 13 kDa (Fig. 3.3B). These data demonstrate that the site of photoaffinity labelling for [^{3}H]flunitrazepam in the cerebellum also occurs prior to residue Asn 103 of the $GABA_A$ receptor α subunit.

Non-specific photoaffinity labelling by [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 in both cortical and cerebellar receptor preparations represented a minor fraction of radioactivity, such that no [^{3}H]-peaks above background levels were detectable in the gel profiles.

pH Dependency of Benzodiazepine Radioligand Binding

The binding of [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 to brain membranes prepared from bovine cerebral cortex under equilibrium conditions was investigated over a pH range from 5 to 9 in a buffer system which controls for ionic strength (see Methods section). Data are expressed as means $\pm$ SEM ($n = 5$) and were calculated to percent specific bound by subtracting non-specific from total bound (non-specific accounted for less than 5% of the total bound) and normalizing to pH 7 as 100%. As seen in Fig. 3.4, the binding of [^{3}H]flunitrazepam is significantly reduced in the pH range from 5 to 7, while the binding of [^{3}H]Ro15-4513 does not significantly deviate from control values over the pH range investigated.

DISCUSSION

Previous studies have investigated the proteins from rat cerebellar and hippocampal membranes that are photoaffinity labelled by [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 and the peptide products that are generated from proteolytic cleavage reactions. A differential degradation of the photoaffinity labelled proteins was demonstrated from proteolytic digestion with papain, trypsin and chymotrypsin (Sieghart et al., 1987a, Sieghart, 1988). However, these reports were unable to conclude if the different cleavage patterns were due to [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 photoaffinity labelling sites on different subunits of the $GABA_A$ receptor or were due to labelling sites on different domains of the same receptor protein(s). Although there is

strong evidence for the α subunit(s) being the major site of labelling by [^{3}H]flunitrazepam (see Chapter 2), similar evidence for the location of the sites of labelling by [^{3}H]Ro15-4513 has not been reported. This study has demonstrated; (1) that the α subunit(s) of the $GABA_A$ receptor is the major site for photoaffinity labelling by both [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 and (2) that the different peptide products generated by hydroxylamine cleavage is due to labelling of distinct domains of this receptor subunit(s).

The hydroxylamine cleavage of [^{3}H]flunitrazepam photoaffinity labelled receptors from cerebral cortex and cerebellum results in a peptide that could only be generated from an α subunit, because the ß, γ and δ subunits do not contain hydroxylamine-specific cleavage sites. Since the α_1 subunit possesses only one potential Asn-Gly peptide bond for cleavage by hydroxylamine at 103/104, the site of the [^{3}H]flunitrazepam photoaffinity label can be further localized to lie within amino acid residues 1 to 103 (α_1 subunit numbering). Previous reports have shown that the [^{3}H]flunitrazepam label occurs on the α subunit and its position is most likely limited to lie within residues 59 to 149 of the α subunit (Stephenson and Duggan, 1989; Olsen et al., 1991). The information gained from the hydroxylamine cleavage pattern extends the earlier predictions and further limits the site of [^{3}H]flunitrazepam photoaffinity label to within α_1 subunit residues 59 and 103. Localization of the [^{3}H]flunitrazepam photoincorporation site to this α-subunit domain corroborates the evidence presented from microsequencing of photoaffinity labelled peptides generated by cyanogen bromide cleavage of bovine $GABA_A$ receptors, which indicated the photolabel is likely to be associated with His 102 of the α_1 subunit (see

Chapter 2). As shown in Fig. 3.4, the involvement of a histidine residue in the binding of the agonist to bovine brain membranes is supported by the decrease in [^{3}H]-flunitrazepam binding in the pH range from 5 to 7. The only amino acid with a pK_a in solution over this range is a histidine residue. The binding of [^{3}H]Ro15-4513 over the pH range from 5 to 7 is not significantly altered. Although previous reports have shown that varying buffer pH has differential effects on the binding of radiolabelled benzodiazepines (see above), the results cannot be unequivocally attributed to pH, since in earlier investigations changes in ionic strength were not controlled and these may have contributed to the observed effects.

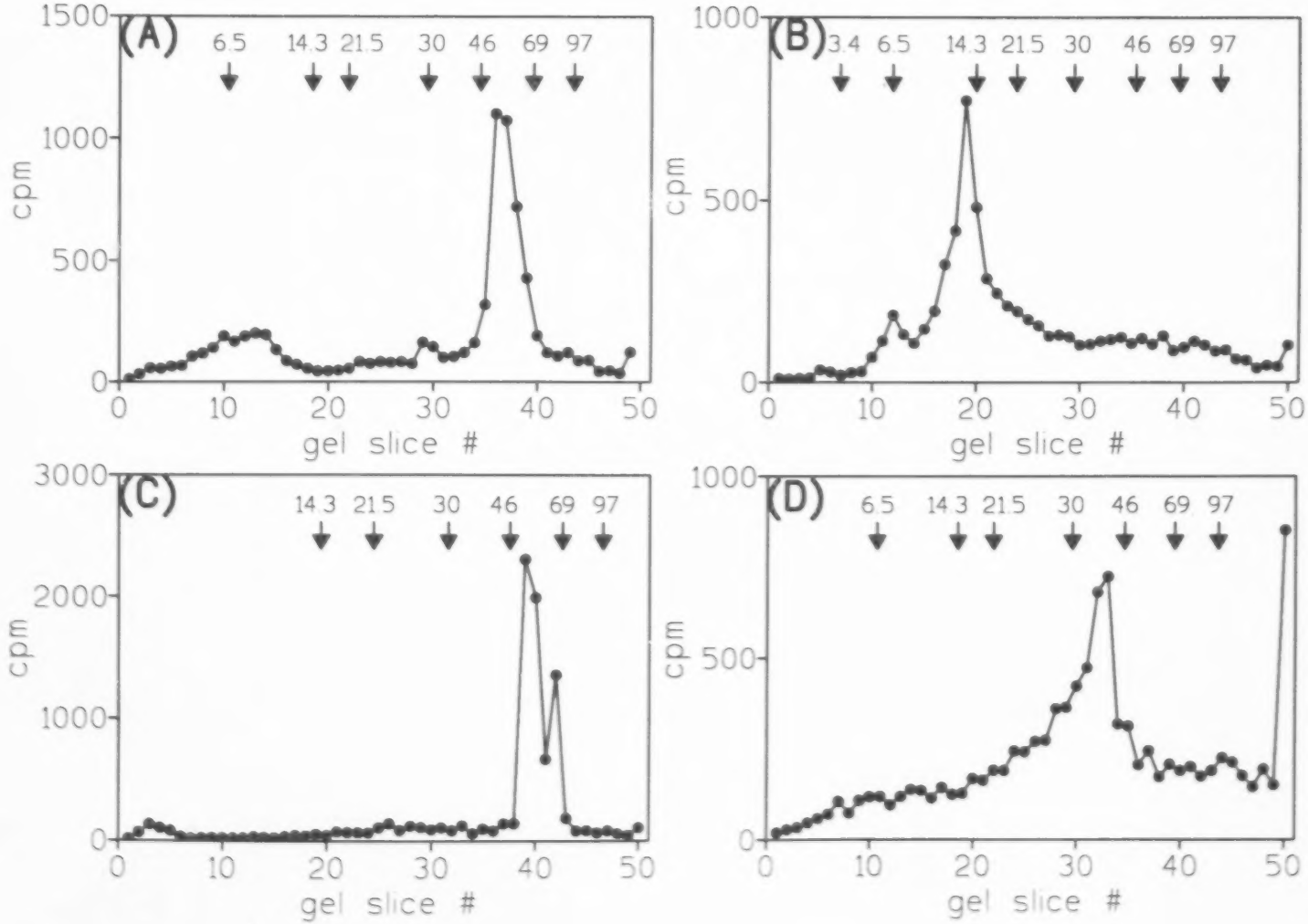
[^{3}H]Ro15-4513 has also been shown to label $GABA_A$ receptor α subunits in cerebral cortex and cerebellum. In the diazepam-sensitive population, this ligand does not label the same fragment as [^{3}H]flunitrazepam, but instead labels the larger Gly 104 - carboxy terminal peptide generated by hydroxylamine cleavage. It is noteworthy that in these hydroxylamine cleaved preparations, there was a small but reproducible portion of radiolabelled product that aggregated at the top of the resolving gel when attempting to resolve by SDS-PAGE. Although it is not possible to make a conclusive explanation for this lack of resolution, it is possible that the removal of a large portion of the amino-terminal domain from the receptor subunit adversely effected the solubility properties of the peptide.

Photoaffinity labelling of the diazepam-insensitive receptors in the cerebellum by [^{3}H]Ro15-4513 occurs in the amino-terminal domain, 1 - 101 (α_6 subunit numbering). Previous reports have presented data that suggested the interaction of [^{3}H]Ro15-4513 with

the diazepam-insensitive receptors was unique compared to the agonist-sensitive population. For example, GABA has been shown to enhance [^{3}H]Ro15-4513 binding to the diazepam-insensitive sites in cultured cerebellar granule cells (Malminiemi and Korpi, 1989), whereas GABA has an inhibitory effect on [^{3}H]Ro15-4513 binding to diazepam-sensitive sites. It is known that the α_6 subunit possesses an arginine in position 100 instead of the histidine residue that is present in the α subunits sensitive to classical benzodiazepine agonists (Wieland et al., 1992). It is tempting to speculate that it is the lack of this histidine residue and/or the presence of an arginine that facilitates [^{3}H]Ro15-4513 binding to this domain of the α_6 subunit.

This study has shown that the sites for photoaffinity labelling by the classical agonist and the partial inverse agonist are located within distinct domains of the α subunit(s). The specific chemical cleavage by hydroxylamine was presumably complete, since intact photolabelled subunit was not evident in the cleavage products from either the [^{3}H]flunitrazepam or [^{3}H]Ro15-4513 receptor preparations. However, residual intact subunit in low abundance may not have been detectable amongst the background radioactivity in the SDS-PAGE profiles. Therefore, while the α-subunit(s) have been established as the major site for photoincorporation by these ligands, the possibility for a minor extent of photolabelling occuring on other non-α subunits cannot be dismissed. Inevitably, the conclusive characterization of the photoaffinity labelling sites of these benzodiazepines on the $GABA_A$ receptor will require complete sequencing of the peptide segments to which the labels are covalently attached.

Figure 3.1. Photoaffinity labelled $GABA_A$ receptors of brain membranes from bovine cerebral cortex and hydroxylamine generated peptides of the labelled receptor preparations. Representative radioactivity profiles of labelled species resolved by Tricine SDS-PAGE are shown. The numerals above each arrow indicate the relative position of the molecular mass standards (expressed in kDa). (A) [^{3}H]flunitrazepam photoaffinity labelled receptor resolves to one major peak that corresponds to a 53 kDa protein band. (B) [^{3}H]flunitrazepam labelled peptides generated by hydroxylamine cleavage yield a major radioactive peak which corresponds to a peptide with an apparent mass of 12 kDa. (C) [^{3}H]Ro15-4513 labelled receptor resolves as a doublet peak with a main 55 kDa protein band and a less abundant 65 kDa species. (D) Hydroxylamine cleaved [^{3}H]Ro15-4513 preparations yield a broad protein band which peaks at an apparent mass of 40kDa. Some labelled protein does not enter into the resolving gel and results in a radioactive peak at the interface (gel slice #50). Data shown are representative at least 2 distinct receptor preparations that have each been resolved by gel electrophoresis a minimum of two times for molecular weight determination.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gel slice #
(B)
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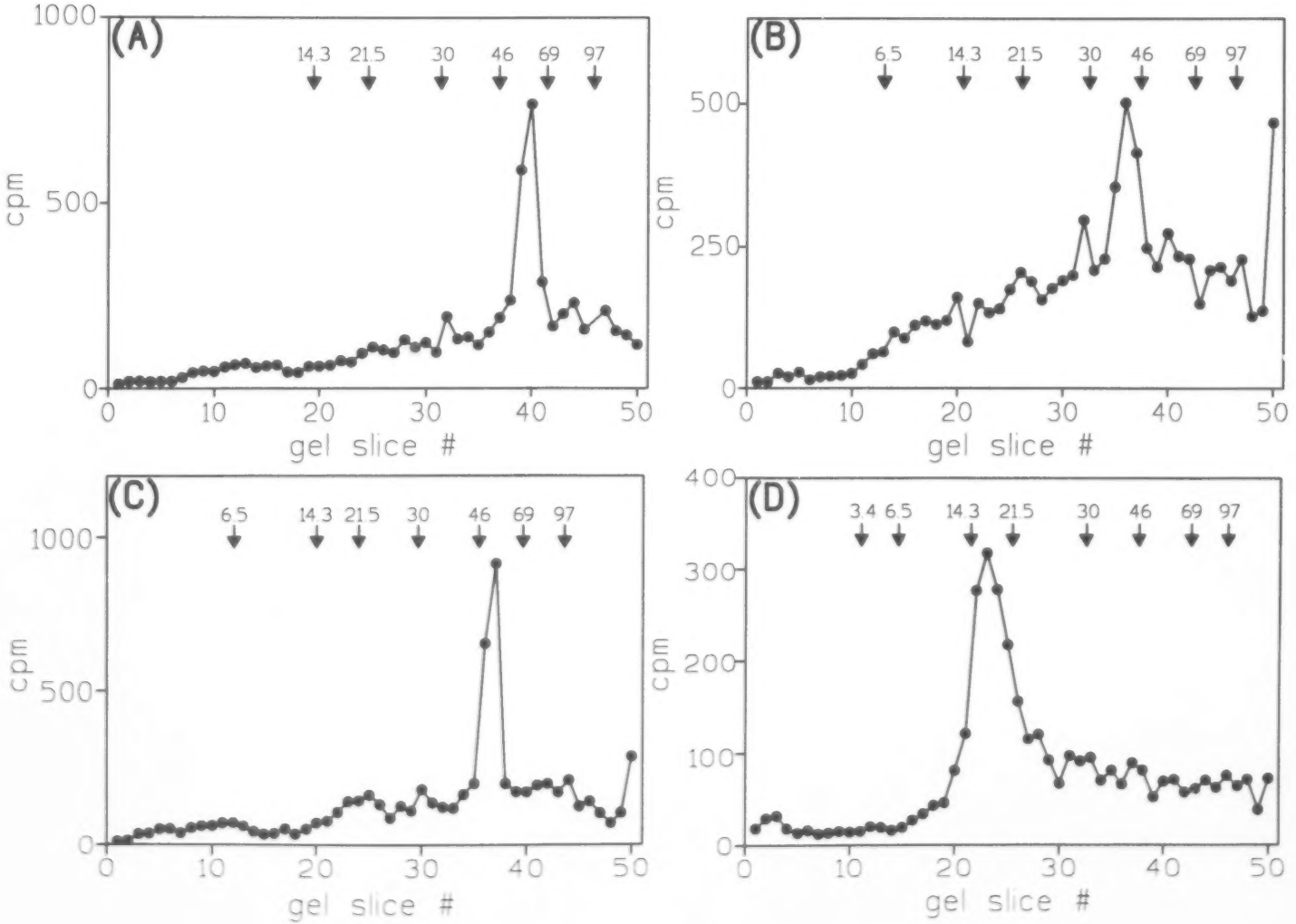
6.5
14.3
21.5
30
46
69
97
1000
500
0
cpm
gel slice #
(C)
14.3
21.5
30
46
69
97
3000
2000
1000
0
cpm
gel slice #
(D)
6.5
14.3
21.5
30
46
69
97
1000
500
0
cpm
gel slice #

Figure 3.2. Representative radioactivity profiles of [^{3}H]Ro15-4513 photoaffinity labelled $GABA_A$ receptors and hydroxylamine generated peptides of bovine cerebellar total sites and diazepam-insensitive sites resolved by SDS-PAGE. (A) The total population of cerebellar sites that are photoaffinity labelled by [^{3}H]Ro15-4513 resolve to a single protein band with an apparent mass of 58 kDa. (B) The hydroxylamine peptides generated from chemical cleavage of the total receptor population resolve to major peak of 43 kDa. Some components of the hydroxylamine reaction of this receptor preparation remain at the top of the resolving gel (peak at gel slice #50). (C) [^{3}H]Ro15-4513 photoaffinity labelled diazepam-insensitive receptor population of the cerebellum resolves to a main 57 kDa band. (D) Photoaffinity labelled peptides generated by hydroxylamine cleavage of the diazepam-insensitive $GABA_A$ receptors resolve to a band with an apparent mass of 14 kDa. Results shown are representative data from at least two preparations that were each resolved by SDS-PAGE two to three times.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1000
500
0
cpm
0 10 20 30 40 50
gel slice #
(B)
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500
250
0
cpm
0 10 20 30 40 50
gel slice #
(C)
6.5 14.3 21.5 30 46 69 97
1000
500
0
cpm
0 10 20 30 40 50
gel slice #
(D)
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cpm
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gel slice #

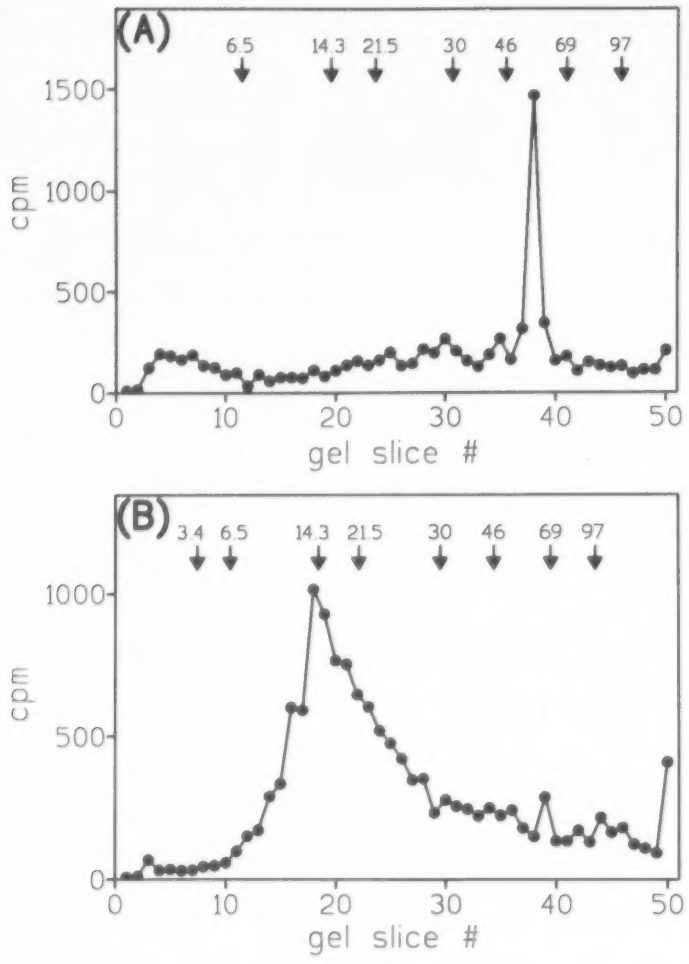


Figure 3.3. [^{3}H]Flunitrazepam photoaffinity labelled receptor of cerebellum and hydroxylamine generated peptides. (A) [^{3}H]flunitrazepam labelled receptor resolves to major radioactive peak of apparent mass of 55 kDa. (B) The hydroxylamine peptides of this preparation resolve to a broad peak with an approximate mass of 13 kDa. Data shown are representative and have been repeated with similar results.

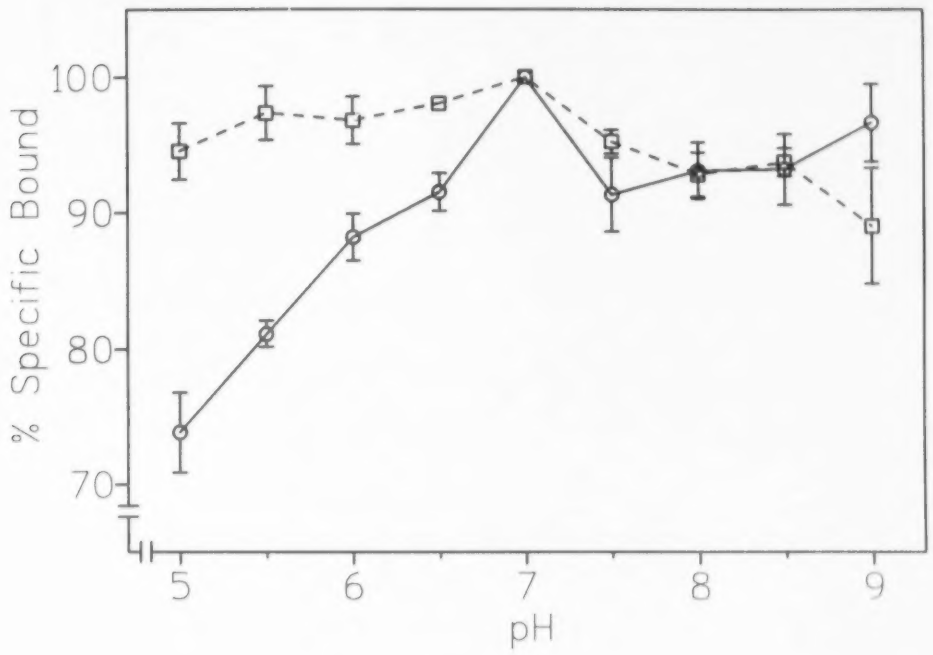


Figure 3.4. Effect of pH on the binding of [^{3}H]FNZ and [^{3}H]Ro15-4513 to brain membranes from cerebral cortex. Binding assays were performed as described in Methods section. Data are expressed as the mean +/- SEM of five separate experiments performed in duplicate. Specific bound for each ligand is normalized to pH 7 of 100%. The binding of [^{3}H]flunitrazepam to brain membranes (○) is significantly reduced below pH 7 compared to control, while [^{3}H]Ro15-4513 binding (□) displays no significant differences across the pH range tested (Student's *t* test, $P < 0.01$).

CHAPTER 4

Benzodiazepine binding to $GABA_A$ receptors: Differential effects of sulfhydryl modification

A version of this chapter has been published. Duncalfe, L.L. and S.M.J. Dunn, 1993, Eur. J. Pharmacol. Mol. Pharmacol. 246, 141.

INTRODUCTION

The classical agonists, inverse agonists and antagonists are subsets of benzodiazepine ligands which interact within a similar binding domain to modulate the $GABA_A$ receptor-chloride channel complex. However, the molecular mechanisms by which the benzodiazepines elicit their actions on the $GABA_A$ receptor and the structural determinants with which they interact have not been fully characterized.

In this study, sulfhydryl modification of the $GABA_A$ receptor(s) in membrane preparations from bovine cerebral cortex has been examined for its effect on the binding of the classical agonist, flunitrazepam, and the partial inverse agonist, Ro15-4513. It is shown that Ro15-4513 binding, much more so than flunitrazepam binding, is sensitive to disulfide reduction, but that the binding is protected by carrying out the reduction in the presence of either benzodiazepine. The binding of the two benzodiazepines can also be distinguished by their different sensitivities to the reaction of $GABA_A$ receptors with N-ethylmaleimide.

MATERIALS AND METHODS

Preparation of Brain Membranes from Bovine Cerebral Cortex

Bovine brain membranes were prepared from cerebral cortex as described previously (see Chapter 2). Prior to use, a membrane aliquot was thawed from -80°C, diluted into the appropriate buffer and recovered by centrifugation. The protein

concentration in the final samples was measured by the method of Bradford (1976) and, unless otherwise stated, the buffer used in all binding assays was 100 mM Tris-HCl, pH 7.4, 0.02% NaN_3.

Binding of Radiolabelled Ligands to Bovine Brain Membranes

Equilibrium binding of [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 (74.1 and 26.0 Ci/mmol, respectively; Dupont Canada) to brain membranes prepared from bovine cerebral cortex was measured in filtration assays using a Hoefer filtration apparatus. In competition experiments, 350μl-aliquots of membranes were added to 350 μl buffer containing either [^{3}H]Ro15-4513 or [^{3}H]flunitrazepam (2.5 nM) and the indicated concentrations of unlabelled flunitrazepam (Sigma Chemicals) or Ro15-4513 (Hoffmann-La Roche) to give a final protein concentration of 1 mg per ml. Non-specific binding was measured in the presence of 1.4 μM unlabelled ligand. After incubation in the dark for 45 min at 4°C, 0.5 ml of each sample was filtered under vacuum through Whatman GF/C filters and the filters were immediately washed with two 5-ml volumes of ice cold buffer. The filters were dried and 5-ml of ACS scintillation fluid (Amersham Canada) was added. The samples were assayed for [^{3}H] by direct scintillation counting. Aliquots of the incubation mixture were also removed and counted for [^{3}H] in order to provide estimates of total ligand added.

In [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam binding assays on brain membranes pretreated with sulfhydryl-modifying agents, the membrane aliquots were added to various concentrations of dithiothreitol or N-ethylmaleimide (Sigma Chemicals).

Following pre-incubation in the dark for 30 min at room temperature, 2.5 nM radiolabelled ligand was added. The mixture was further incubated in the dark for 45 min at 4°C, then filtered under vacuum as described above. [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam binding assays with dithiothreitol pre-treated membranes under conditions of desensitization were performed in a similar manner, except that 10 μM of the GABA analogue, muscimol (Sigma Chemicals), was included with the membranes and dithiothreitol in the pre-incubation mixture.

To assess a potential direct interaction between the sulfhydryl-modifying agents and the [^{3}H]benzodiazepine ligands, thin layer chromatography of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam in the absence and presence of dithiothreitol or N-ethylmaleimide was performed on Silica gel using a methylene chloride:methanol (9:1) solvent system. The mobility of the ligands was unaffected by incubation with either agent.

Equilibrium binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to bovine cortical membrane preparations for determination of dissociation constants (K_D) and density of binding sites (B_{MAX}) were also measured by a filtration assay. Membrane aliquots (350 μl) were added to different concentrations of radiolabelled ligand to give a final volume of 700 μl and a protein concentration of 1 mg/ml. Non-specific binding was measured in the presence of 1.4 μM unlabelled ligand. Following incubation in the dark for 45 min at 4°C, the samples were filtered and assayed for [^{3}H] as described above. When investigating the effects of dithiothreitol and N-ethylmaleimide on binding parameters for [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam binding, the sulfhydryl-modifying agent was included at a concentration of 10 mM in the mixture and incubated in the dark for 30

min at room temperature and 45 min at 4°C, after which bound and free ligand was separated by filtration. Parallel control samples were also prepared which did not contain dithiothreitol or N-ethylmaleimide.

Protection of Brain Membranes from Dithiothreitol Reduction by Pre-incubation with Benzodiazepine Ligands

Four 25-ml membrane aliquots (protein concentration 1 mg/ml) were incubated in the dark for 45 min at 4°C with either (a) 10 μM unlabelled Ro15-4513, (b) 10 μM unlabelled flunitrazepam, or (c) and (d) an equal volume of buffer. Dithiothreitol was added to a final concentration of 10 mM to samples (a), (b) and (c). To sample (d), an equal volume of buffer was added. The mixtures were incubated for 30 min at room temperature, then centrifuged at 40,000 rpm in a Beckman type 70 Ti rotor for 45 min. The membranes were resuspended with a Potter S homogenizer in 25 ml of 100 mM Tris-HCl pH 7.4, 0.02% NaN_3 containing 0.1 mM dithiothreitol. This concentration of dithiothreitol was included to maintain reducing conditions, but was present at a sufficiently low concentration so as not to interfere with subsequent binding assays. Following an incubation in the dark for 30 min at 4°C, the samples were repelleted. The membranes were subjected to this cycle of resuspension and repelleting two more times to ensure the complete removal of unlabelled ligand and excess dithiothreitol. The membranes were finally resuspended at a protein concentration of 2 mg/ml. Equilibrium measurements of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam binding to the membrane samples were performed by filtration assay as described above.

Statistical Analysis of Binding Data

The data obtained from equilibrium binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to membrane preparations for determination of K_D and B_{MAX} are expressed in the Results section as the mean $\pm$ S.E.M. For binding data obtained in the presence of a sulfhydryl-modifying agent, significance levels (P) were determined with the Student's *t* test by comparison to the corresponding control. The results obtained from [^{3}H]Ro15-4513 binding to membrane samples in the protection from reduction experiments were analysed by a one-way analysis of variance and Duncan's new multiple range test at 95% confidence limits (Dowdey and Weardon, 1983).

RESULTS

Specificity of [^{3}H]Benzodiazepine Binding to Brain Membranes prepared from Bovine Cerebral Cortex

It has previously been reported that, in some brain preparations, there are diazepam-insensitive sites that bind [^{3}H]Ro15-4513 with high affinity (Sieghart et al., 1987b; Turner et al., 1991). The specificity of the high affinity benzodiazepine binding sites in bovine brain cortical membrane preparations has been demonstrated by the ability of unlabelled flunitrazepam and Ro15-4513 to displace bound radiolabelled ligand. Examples of such competition curves are shown in Fig. 4.1 and illustrate that the membranes used in this study do not contain a significant population of diazepam-

insensitive binding sites.

Effects of Dithiothreitol on the Binding of [^{3}H]Ro15-4513 and [^{3}H]Flunitrazepam to Bovine Brain Membranes

The equilibrium binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to bovine brain membranes is differentially affected by dithiothreitol reduction. As shown in Fig. 4.2A, binding of the partial inverse agonist, [^{3}H]Ro15-4513, was significantly decreased by 300 μM dithiothreitol and almost completely abolished at a concentration of 100 mM (IC_{50} of 4.6 mM). However, high concentrations of dithiothreitol (> 30 mM) only moderately inhibited the binding of the classical agonist, [^{3}H]flunitrazepam. These results indicate that there exists a structural requirement of an intact disulfide bridge(s) necessary for the high affinity binding of Ro15-4513 but not for that of flunitrazepam. The differential effect of dithiothreitol on benzodiazepine binding was unaffected by receptor desensitization (Fig. 4.2B). $GABA_A$ receptors were desensitized by incubation of the membranes with 10 μM muscimol. In control experiments, we have shown that dithiothreitol, at concentrations up to 100 mM, has no significant effect on [^{3}H]muscimol binding (data not shown).

The effects of dithiothreitol (10 mM) on the parameters for [^{3}H]flunitrazepam and [^{3}H]Ro15-4513 binding have been investigated in more detail, as shown in the Scatchard plots in Fig. 4.3. In the case of [^{3}H]Ro15-4513, the presence of dithiothreitol caused a reduction in binding affinity by more than three-fold (from 12 $\pm$ 2 nM to 40 $\pm$ 7 nM, $P<0.01$, $n = 4$) and a reduction in the density of binding site from 5.8 $\pm$ 0.4 to 3.7 $\pm$

0.4 pmol/mg ($P<0.01$). Dithiothreitol (10 mM) had no significant effect on either the affinity for [^{3}H]flunitrazepam binding (K_D 6.2 $\pm$ 0.8 nM) or the concentration of its binding sites (2.6 $\pm$ 0.3 pmol/mg) when compared to control values (K_D 5.4 $\pm$ 0.5 nM; B_{MAX} 3.0 $\pm$ 0.1 pmol/mg).

Ligand Protection of Brain Membranes from the Effects of Dithiothreitol Reduction

The involvement of an intact disulfide bridge in Ro15-4513 binding was also demonstrated by the ability of unlabelled Ro15-4513 to protect against the inhibitory effect of dithiothreitol when this ligand was included during the reduction step (Fig. 4.4). After reduction in the presence of 10 μM Ro15-4513 and extensive washing to remove unlabelled ligand, neither the dissociation constant for [^{3}H]Ro15-4513 binding (14.8 $\pm$ 1.4 nM) nor the density of binding sites (4.8 $\pm$ 0.3 pmol/mg) were significantly different from the control values (K_D 14.8 $\pm$ 0.3 nM; B_{MAX} 5.1 $\pm$ 0.2 pmol/mg). In the same experiment, however, dithiothreitol reduction in the absence of unlabelled ligand displayed the expected alterations in [^{3}H]Ro15-4513 binding by both increasing the dissociation constant to 20.1 $\pm$ 0.8 nM and reducing the binding site density to 3.6 $\pm$ 0.2 pmol/mg ($P<0.05$). Thus occupancy of the Ro15-4513 binding site(s) appears to protect the crucial disulfide bridge(s) from reduction. Carrying out the reduction in the presence of 10 μM flunitrazepam also afforded protection, although less complete, against the deleterious effect of dithiothreitol on subsequent [^{3}H]Ro15-4513 binding (data not shown).

Effect of N-ethylmaleimide on [^{3}H]Ro15-4513 and [^{3}H]Flunitrazepam Binding

To further investigate the effects of sulfhydryl modification on benzodiazepine binding, membranes were pretreated with various concentrations of the sulfhydryl alkylating agent, N-ethylmaleimide, and then assayed for radiolabelled benzodiazepine binding. The data illustrated in Fig. 4.5 demonstrate that N-ethylmaleimide inhibits the binding of both ligands with a similar concentration dependence, giving IC_{50} estimates of 13.0 mM for [^{3}H]Ro15-4513 and 20.0 mM for [^{3}H]flunitrazepam binding. Scatchard plots of equilibrium binding data for [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam binding in the absence or presence of 10 mM N-ethylmaleimide showed that the effect of N-ethylmaleimide was to increase the K_D for [^{3}H]Ro15-4513 binding from 12.1 $\pm$ 0.8 to 18.5 $\pm$ 1.3 nM ($P<0.02$), and to reduce the binding site density by 28% from 5.0 $\pm$ 0.2 to 3.6 $\pm$ 0.4 pmol/mg ($P<0.02$). However, although N-ethylmaleimide increased the K_D for [^{3}H]flunitrazepam binding by more than two-fold (from 5.1 $\pm$ 0.7 to 13 $\pm$ 1 nM, $P<0.01$), the density of high affinity [^{3}H]flunitrazepam binding sites was unaffected (Control 3.3 $\pm$ 0.2; N-ethylmaleimide 2.9 $\pm$ 0.1 pmol/mg). Thus, as described for the effects of dithiothreitol on benzodiazepine binding, the alkylating reagent, N-ethylmaleimide, also has different effects on the binding of the two benzodiazepines.

DISCUSSION

Investigations in recent years utilizing a variety of methodological approaches have identified some important structural components required for benzodiazepine modulation of $GABA_A$-mediated responses (see Chapter 1). In the present study, we have investigated the importance of cysteine/cystine residues in benzodiazepine binding and demonstrate that sulfhydryl modification has different effects on the binding of a classical benzodiazepine agonist and a partial inverse agonist.

The results of binding experiments to bovine brain membranes reduced with dithiothreitol demonstrate that the binding of [^{3}H]Ro15-4513 requires an intact disulfide bridge(s) for high affinity binding, whereas reduction of this bond(s) does not affect [^{3}H]flunitrazepam binding. These contrasting effects of dithiothreitol cannot be explained by interaction of the benzodiazepine ligands with different $GABA_A$ receptor subtypes, since these membranes did not possess any significant population of diazepam-insensitive binding sites. Additionally, flunitrazepam competitively inhibited all of the [^{3}H]Ro15-4513 binding, as did Ro15-4513 inhibit all of the [^{3}H]flunitrazepam binding. Although the binding of the two ligands appears to be mutually competitive, it is evident that the density of binding sites for [^{3}H]Ro15-4513 differs from that of [^{3}H]flunitrazepam binding. Previous studies using rat brain membranes prepared from cerebral cortex have also reported discrepancies in binding site density for these benzodiazepine ligands (Binkley and Ticku, 1991) and comparable results for the number of [^{3}H]Ro15-4513 binding sites in bovine cortical membranes (approximately 5 pmol/mg protein) were found by Turner

et al. (1991). However, the results presented here demonstrate that differential effects of dithiothreitol reduction cannot be explained by the discrepancy in number of sites. Not only do the sites for Ro15-4513 and flunitrazepam appear to be mutually exclusive, but the results shown in Fig. 4.2 show no evidence for heterogeneity in the effects of dithiothreitol on [^{3}H]Ro15-4513 binding, as would be expected if dithiothreitol was affecting only the "additional" sites for this ligand. The Scatchard plots in Fig. 4.3 also suggest that the binding of [^{3}H]Ro15-4513 is to a homogeneous population of sites, both in the presence and absence of dithiothreitol. The effects of dithiothreitol were unaffected by desensitization of the $GABA_A$ receptor(s) induced by prolonged exposure to the agonist, muscimol (Krnjevic, 1981). Thus, the intact disulfide bond(s) that is crucial for Ro15-4513 binding is sensitive to reduction in either the resting or desensitized conformation of the receptor protein. Investigation of the mechanism by which dithiothreitol inhibits [^{3}H]Ro15-4513 binding has revealed that both the affinity and number of binding sites are significantly reduced after disulfide reduction. In contrast, the binding of [^{3}H]flunitrazepam was affected only by higher concentrations of dithiothreitol (greater than 30 mM). This relative insensitivity to reduction is in agreement with an earlier report (Korneyev et al., 1985) in which 50 mM dithiothreitol caused only a slight change in the dissociation constant (less than 2-fold) for [^{3}H]flunitrazepam without affecting the density of sites in several areas from bovine brain.

The ability of unlabelled benzodiazepines to protect the disulfide(s) that are important for Ro15-4513 binding provides further evidence for the presence of a disulfide

bridge that is either in close proximity to the benzodiazepine binding domain or is involved in stabilizing the conformation of the binding site. Occupancy of benzodiazepine binding sites by Ro15-4513 during the reduction negated the inhibitory effects of dithiothreitol on [^{3}H]Ro15-4513 binding. This protective effect may be interpreted either by steric occlusion of a nearby disulfide(s) by the presence of the ligand, or by a mechanism whereby occupancy of the benzodiazepine binding sites induces a receptor conformation that reduces the reactivity of the disulfide(s). The presence of flunitrazepam during reduction also provided some protection of the disulfide involved in partial inverse agonist binding, suggesting commonalities in the binding sites for the two benzodiazepine ligands or in the receptor conformations that they induce.

All known subunits of the ligand-gated ion channel family contain a highly conserved pair of cysteine residues which are presumed to form a ß-structural loop and have been predicted to play a role in stabilizing the tertiary structure of the receptor (see Chapter 1). This disulfide has been implicated in α-bungarotoxin binding to the nicotinic acetylcholine receptor (Sumikawa & Gehle, 1992). While it has not been confirmed that it is the reduction of this disulfide bond which results in the loss of high affinity binding for the partial inverse agonist, this pair of cysteines are the only residues available in the amino-terminal domain of the $GABA_A$ receptor subunits to form a disulfide bond.

The binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam also displayed differences in their sensitivity to N-ethylmaleimide. It was previous reported (Zundel et al., 1985) that N-ethylmaleimide (1 mM) slightly potentiated (by about 9%) the binding of [^{3}H]flunitrazepam to rat hippocampal membranes, but higher concentrations were not

investigated. In the present study, N-ethylmaleimide, at concentrations up to 1 mM, had little effect on either [^{3}H]flunitrazepam or [^{3}H]Ro15-4513 binding but the binding of both benzodiazepines was inhibited at higher concentrations. More detailed analysis revealed that both the affinity and number of binding sites for [^{3}H]Ro15-4513 were reduced, whereas only the affinity for [^{3}H]flunitrazepam was affected. Thus both a reducing agent and an alkylating agent have similar effects on the binding of [^{3}H]Ro15-4513 suggesting that both a disulfide bond and a free sulfhydryl group may be important for stabilizing the conformation of the binding site, as has been shown, for example, for the binding of agonists to the nicotinic acetylcholine receptor (Moore and Raftery, 1979).

In conclusion, it has been demonstrated that sulhydryl groups are involved in the benzodiazepine interaction with the $GABA_A$ receptor and that an intact disulfide bond is required for the binding of Ro15-4513, but not for flunitrazepam binding.

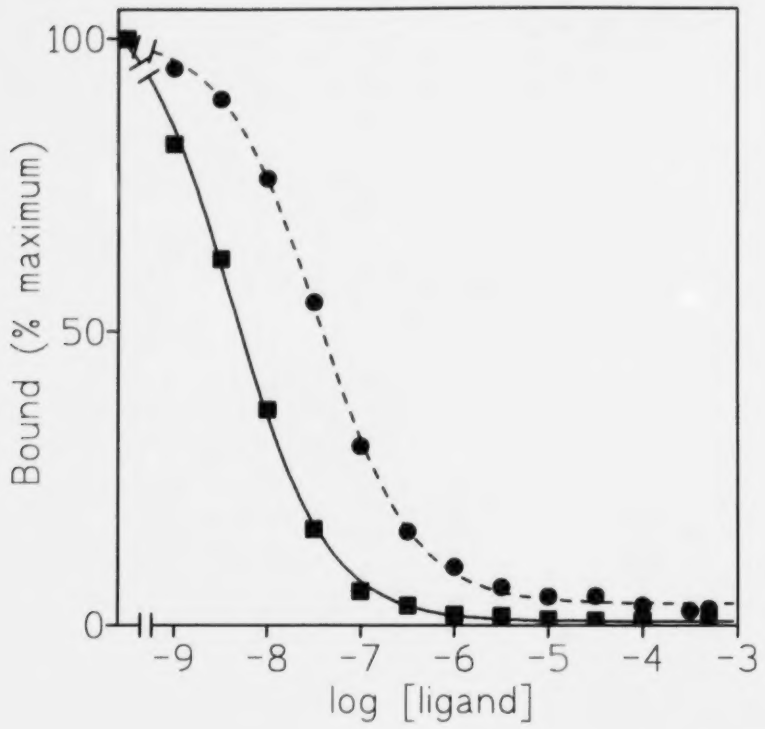


Figure 4.1. Effects of unlabelled benzodiazepine ligands on the binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to bovine brain membranes. Membranes (1 mg protein per ml) were incubated with 2.5 nM [^{3}H]Ro15-4513 and various concentrations of flunitrazepam (■) or 2.5 nM [^{3}H]flunitrazepam and various Ro15-4513 concentrations (●) for 45 min at 4°C, after which bound radiolabel was estimated by filtration. Data shown are representative of 2 separate experiments with different membrane preparations. Data are fit to a simple competitive model which gave values for IC_{50} of 4.9 nM (■) and 36.7 nM (●).

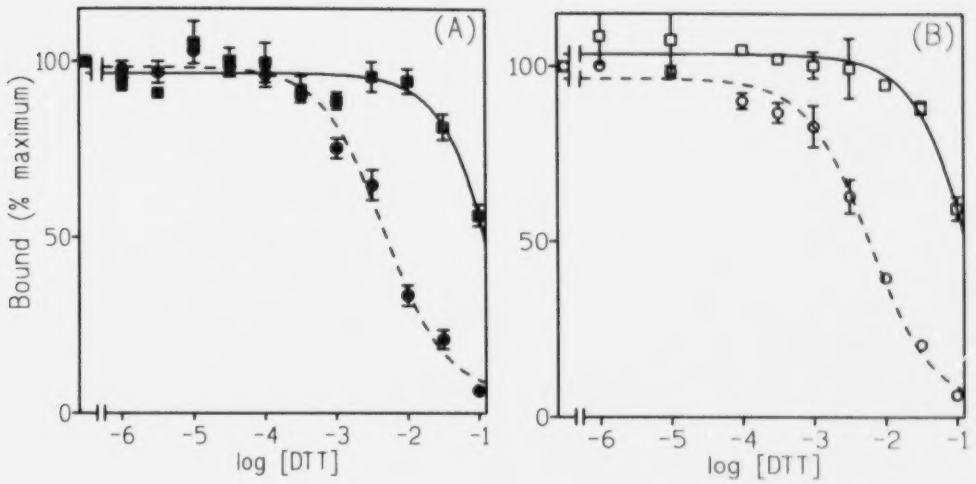


Figure 4.2. Effects of dithiothreitol (DTT) on the binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to bovine brain membranes. (A) Membranes (1 mg protein per ml) were incubated with increasing concentrations of dithiothreitol for 30 min at room temperature before adding 2.5 nM [^{3}H]Ro15-4513 (●) or [^{3}H]flunitrazepam (■). Following further incubation for 45 min at 4°C, bound and free ligand were separated by filtration. Results are expressed as the means ± S.E.M. of three separate experiments. Estimated IC_{50} values were 4.6 mM (●) and 131 mM (■). (B) The effects of dithiothreitol on the binding of [^{3}H]Ro15-4513 (○) and [^{3}H]flunitrazepam (□) to membranes desensitized with muscimol. Membranes were treated as described for (A), but 10 μM muscimol was included in the first incubation mixture. Data shown are the averages ± S.E.M. from 2 different experiments. Best fit values for IC_{50} were 5.9 mM (○) and 165 mM (□).

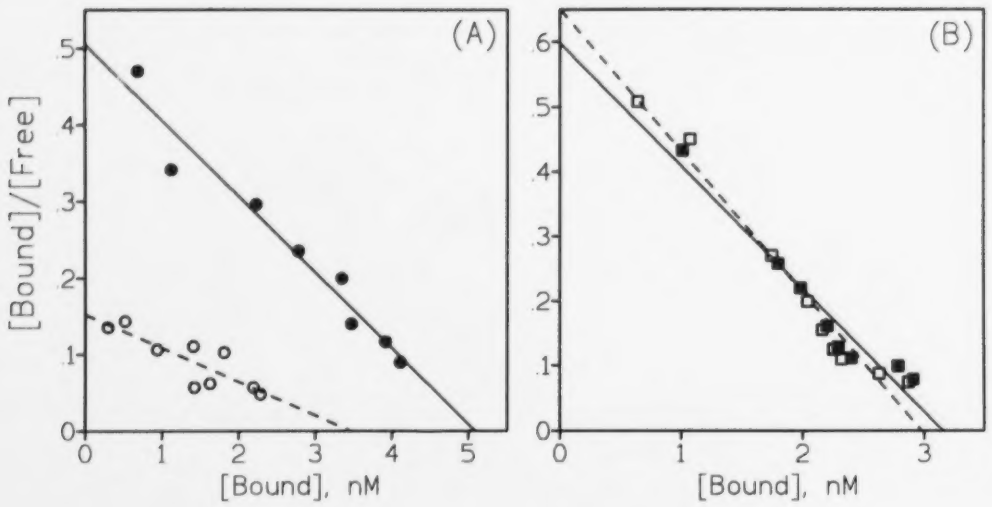


Figure 4.3. Equilibrium binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to bovine brain membranes in the presence of dithiothreitol. (A) Scatchard plots of [^{3}H]Ro15-4513 binding in the absence (●) or presence (○) of 10 mM dithiothreitol. Linear least squares fit gave values for K_D and B_{MAX} of 10.1 nM, 5.1 pmol/mg (●) and 22.7 nM, 3.5 pmol/mg (○), respectively. (B) Scatchard plots of [^{3}H]flunitrazepam binding in the absence (■) or presence (□) of 10 mM dithiothreitol, with best fit values for K_D and B_{MAX} of 5.3 nM, 3.2 pmol/mg (■) and 4.6 nM, 3.0 pmol/mg (□), respectively. The results shown are representative data from 4-6 experiments. The amount bound is expressed as concentration (nM) and since the protein concentration was 1 mg/ml, 1 nM = 1 pmol/mg.

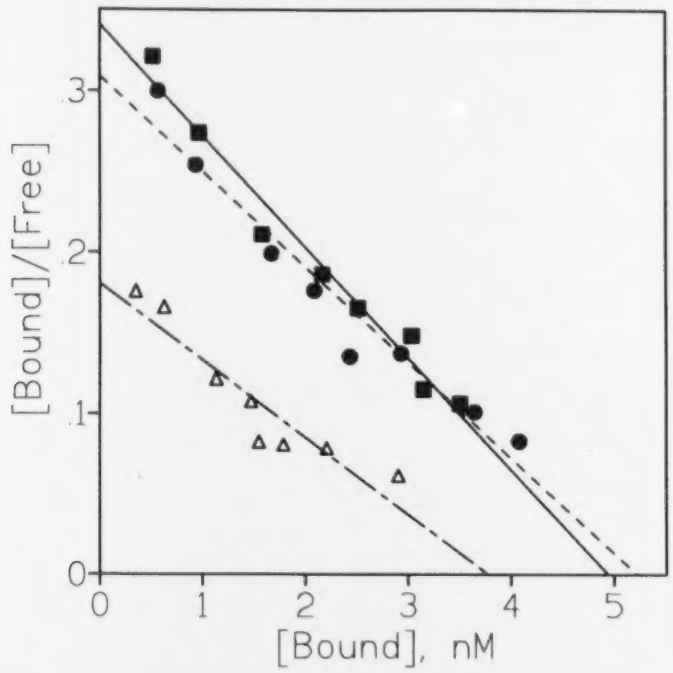
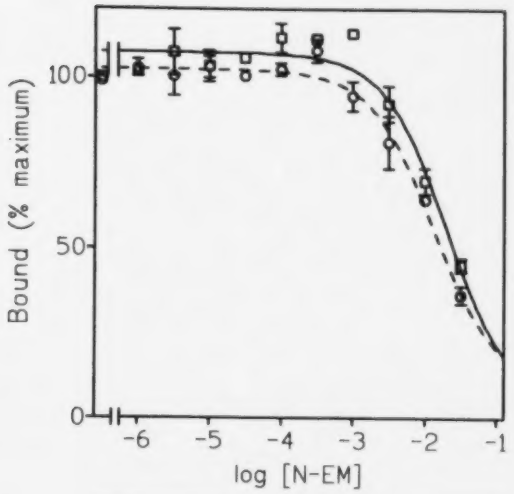


Figure 4.4. [^{3}H]Ro15-4513 binding to bovine brain membranes: protection from the effects of dithiothreitol by the presence of unlabelled Ro15-4513 during reduction. Equilibrium binding data of [^{3}H]Ro15-4513 to control membranes (■), to membranes reduced with 10 mM dithiothreitol (▵), and to membranes pre-incubated with 10 μM Ro15-4513 prior to reduction with 10 mM dithiothreitol (●) are presented as Scatchard plots. Results shown are representative of data from 3 different experiments. Best fit values for K_D of (■), (▵), and (●) are 14, 21, and 16 nM and for B_{MAX} are 4.9, 3.8, and 5.2 pmol/mg (respectively).

Figure 4.5. Effects of N-ethylmaleimide on the binding of [^{3}H]Ro15-4513 and [^{3}H]flunitrazepam to bovine brain membranes. (A) Experimental procedures were as in the legend to fig. 4.2A except that membranes were added to different concentrations of N-ethylmaleimide. Results are expressed as the averages ± S.E.M. from 3 distinct experiments. IC_{50} values were estimated to be 13.0 mM for [^{3}H]Ro15-4513 (○) and 20.0 mM for [^{3}H]flunitrazepam (□). (B) Scatchard plots of [^{3}H]Ro15-4513 binding to membranes in the absence (●) or presence (○) of 10 mM N-ethylmaleimide. Linear least squares regression gave values for K_D and B_{MAX} are 12.0 nM, 5.1 pmol/mg (●) and 17.8 nM, 2.9 pmol/mg (○), respectively. (C) Scatchard plots of [^{3}H]flunitrazepam binding to membranes in the absence (■) or presence(□) of 10 mM N-ethylmaleimide. Best fit values for K_D and B_{MAX} are 4.0 nM, 2.8 pmol/mg (■) and 12.7 nM, 2.8 pmol/mg (□), respectively. Results shown are representative data from 3 experiments.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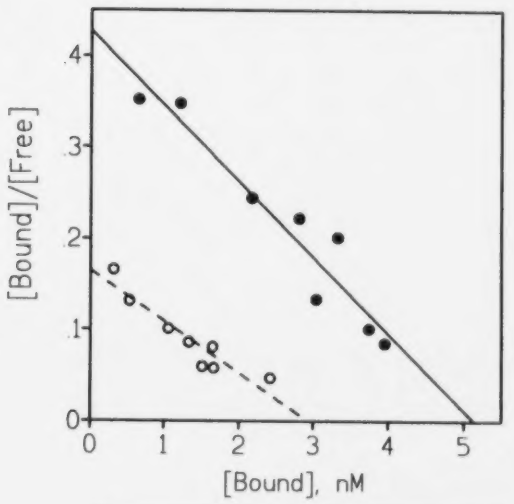
50
0
-6
-5
-4
-3
-2
-1
log [N-EM]


[Bound]/[Free]
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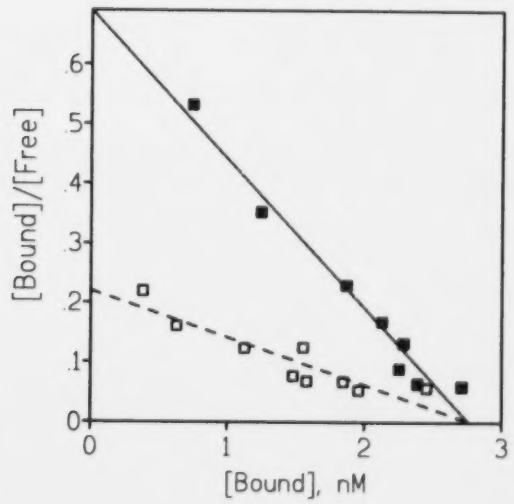
.3
.2
.1
0
0
1
2
3
4
5
[Bound], nM


[Bound]/[Free]
.6
.5
.4
.3
.2
.1
0
0
1
2
3
[Bound], nM

CHAPTER 5

General Discussion and Conclusions

GENERAL DISCUSSION

To understand the mechanisms by which neuropharmacological agents alter brain function, their interactions at all levels of organization of the central nervous system must be considered (Haefely, 1994). Centrally-active ligands interact with specific receptors to effect a cascade of events that may begin with neurotransmission at a single synapse, but lead to the modulation of cells involved with signal transduction through complex neuronal networks, with far reaching effects on physiological responses and behavior.

Based mainly on the physicochemical properties of GABA, some have declared it to be the "quintessential" neurotransmitter (Roberts and Sherman, 1993). While this remains open for debate, it is generally agreed that the $GABA_A$ receptor is distinctive because of several discrete modulatory sites that display complex allosteric interactions on the multisubunit protein. In particular, the benzodiazepine site is unique because of the diversity of ligands that interact with this site to elicit diametrically opposed actions. The identification of the structural determinants that participate in the interaction of the benzodiazepines with the $GABA_A$ receptor is an important and necessary step for elucidating the mechanisms of action of these ligands. The study of the allosteric modulation of $GABA_A$ receptors by benzodiazepine ligands will inevitably contribute to our understanding of the plasticity of neurotransmitter receptors and, more generally, of the mechanisms of interaction of small molecules with proteins.

Benzodiazepines interact with the $GABA_A$ receptor and, since GABA is the major inhibitory neurotransmitter in the vertebrate central nervous system, the number of

synapses that are modulated by these ligands is very large, i.e. one third to one half of all central synapses (Sieghart, 1995). The potential benefits of designing therapeutic agents with the ability to target specific $GABA_A$ receptor subtypes and therefore to have effects on specific physiological responses are significant. Due to the ubiquity of GABA in the brain, benzodiazepines can influence a multitude of behavioral effects. These include circadian rhythms, appetite and food intake, motor function, sexual reproduction, aggressive-defensive behaviors, cognition, vigilance and memory (Haefely, 1994). A highly selective behavioral action would result from a benzodiazepine that had the ability to target a specific subset of $GABA_A$ receptors that was responsible for mediating a particular effect. Understanding the fundamental structural features involved in the interaction of different benzodiazepine ligands with $GABA_A$ receptor subtypes in various brain regions is integral to achieving selective enhancement of desired pharmacological effects.

Elucidation of the mechanisms of action of benzodiazepines with diverse efficacies may also facilitate the development of selective agonists that lack the propensity to induce functional tolerance. Prolonged exposure to classical benzodiazepine agonists often results in the development of functional tolerance to some of their actions (see Sieghart, 1995). While this tolerance is known to affect the allosteric interactions between the receptor and the benzodiazepine binding site, the molecular basis for the "uncoupling" and decreased benzodiazepine efficacy has not been established (Sieghart, 1995). Interestingly, partial agonists at the benzodiazepine site fail to induce functional tolerance. Therefore, a benzodiazepine which is subtype selective and a partial agonist

would display the advantageous clinical effects of full agonists, but have selective efficacy with reduced dependence and abuse potential (Costa et al., 1994). In addition, full inverse agonists at the benzodiazepine site exhibit convulsant and anxiogenic actions and these have been studied for purely scientific interest. However, partial inverse agonists interacting with distinct receptor subtypes that act as mild stimulants of arousal, attention and cognition may be desirable from a therapeutic standpoint. Again, knowledge of ligand interactions at a molecular level may provide the information to design drugs with increased specificity of action.

The present information on both the GABA and benzodiazepine binding sites of the $GABA_A$ receptor is insufficient to construct a detailed model of the protein domains that initiate conformational changes to control channel function. However, due to the homology of $GABA_A$ receptor binding sites with those of other ligand-gated ion channels, analogies can be drawn to other members of the receptor superfamily (see below). Interest is focused on the nicotinic acetylcholine receptor, due to the extensive chemical characterization of the receptor that has benefited from the large amounts of receptor protein available from *Torpedo* electric organ (reviewed by Galzi and Changeux, 1995). Structural and functional studies of the nicotinic acetylcholine receptor have provided detailed information on quaternary organization, on the molecular mechanisms of the interactions between various ligand-binding sites and on the regulation of the integral ion channel.

In the nicotinic receptor, the binding sites for agonists appear to be formed by several segments of the extracellular N-terminal domain of each subunit, but residues at

subunit - subunit interfaces also appear to be important determinants of both agonist and competitive antagonist binding (reviewed by Conti-Tronconi et al., 1994). The evidence from photoaffinity labelling and heterologous expression studies suggest that the agonist site on $GABA_A$ receptors may also occur at an interface between two dissimilar subunits (see Dunn et al., 1994). Although the benzodiazepines are not agonists at the GABA site on the $GABA_A$ receptor, the structural properties of their binding domain display many similarities to the agonist sites of ligand-gated ion channels. For example, Tyr93 of the α-subunit has been implicated in acetylcholine binding to the nicotinic receptor (Galzi et al., 1990). This amino acid residue is analogous to His102 of the $GABA_A$ receptor α-subunit, the residue that is fundamental for the interaction of benzodiazepine agonists with the receptor.

Based on the degree of homology seen for the ligand binding domains of GABA and benzodiazepines, it has been proposed that an agonist site on the $GABA_A$ receptor may have evolved, through gene duplication, into the allosteric modulatory site now seen for the benzodiazepines (Smith and Olsen, 1995). Indeed, available evidence suggests that the benzodiazepine site may involve protein domains similar to the GABA sites on other subunits and may also occur at the subunit interfaces. Therefore, the molecular mechanisms utilized by the benzodiazepines for GABA-mediated neuromodulation may represent general schemes of ligand-protein interactions which extend to other members of the ligand-gated ion channel superfamily.

CONCLUSIONS

Several structural determinants required for the allosteric modulation of $GABA_A$ receptors by the benzodiazepines have been characterized by site-directed mutagenesis of recombinant receptors in heterologous expression systems (see Introduction). However, it has yet to be determined if recombinantly expressed receptors accurately represent the native population of $GABA_A$ receptors in the mammalian brain. Further, the interpretation of site-directed mutagenesis is complicated, since the identified amino acid residues are not necessarily directly involved in benzodiazepine binding, but rather may play structural roles for the maintenance of protein conformation required for ligand interactions. The other approach that has been used extensively to identify specific amino acid residue(s) that are involved in benzodiazepine binding has been to photoaffinity label the binding site with photoactivatable benzodiazepines, such as flunitrazepam and Ro15-4513. It is uncertain whether these ligands photoincorporate into residues that are integral to the benzodiazepine binding domain or exist in close proximity to the binding site. Lastly, chemical modification of $GABA_A$ receptor proteins with a variety of reagents has been utilized to identify structural features important for ligand-receptor interactions, although findings from these studies implicate the involvement of particular amino acid residues in only a general manner. Despite these caveats, these studies have provided valuable information and the benzodiazepine binding domain has been modelled as a composite of several structural features, using the information gained from heterologous expression, affinity labelling and chemical

modification studies (Smith and Olsen, 1995).

The modelled binding domain for benzodiazepine agonists on the $GABA_A$ receptor includes amino acid residues His102 and Gly200 of the α_1-subunit and Thr142 of the γ-subunit(s). By point mutation of recombinantly expressed receptors, the α_1-subunit Gly200 and γ-subunit Thr142 were identified as important residues for conferring the differential binding characteristics of benzodiazepines and their modulatory actions. The involvement of α_1-subunit His102 was based on earlier reports that used chemical modification techniques to implicate a role for histidine residues in agonist binding (Lambolez and Rossier, 1987; Lambolez et al., 1989), site-directed mutagenesis that specifically showed His102 of α_1 subunit was necessary for the high affinity binding of benzodiazepine agonists (Wieland et al., 1992), and preliminary microsequencing data that showed [^{3}H]flunitrazepam likely photoincorporates with His102 of the α_1 subunit (Olsen and Smith, 1994). The evidence presented in Chapter 2, based on peptide mapping and microsequence analysis of purified photolabelled peptides, confirms that a major site of photoaffinity labelling by [^{3}H]flunitrazepam is His102 of the α-subunit(s). Thus, this residue is predicted to have an integral role in the formation of the benzodiazepine agonist binding domain.

Previous studies have established that Ro15-4513 binding does not require the crucial histidine residue (His102) involved in benzodiazepine agonist and antagonist binding (Binkley and Ticku, 1991; Wieland et al., 1992; Maksay, 1992; Uusi-Oukari, 1992). This information has been extended and the binding domain for the partial inverse agonist on the $GABA_A$ receptor has been shown to have some structural

characteristics that are distinct from the classical agonist site. The site for photoaffinity labelling by [^{3}H]Ro15-4513 on diazepam-sensitive $GABA_A$ receptors occurs after α-subunit(s) residue 104 (Chapter 3). Further, an intact disulfide bond is required for the high affinity binding of the partial inverse agonist (Chapter 4) and the Cys-Cys amino acid pair at position 139/153, presumed to form a disulfide bridge, occurs within the subunit domain that is localized by the photolabelling studies. In addition, a distinctive [^{3}H]Ro15-4513 photoaffinity labelling site is found for the cerebellar diazepam-insensitive $GABA_A$ receptors, which are known to contain the α_6 subunit isoform which lacks the His residue at homologous position 100.

To summarize the major findings of the studies that have been presented here, it has been demonstrated that; (1) the classical agonist, flunitrazepam, photoincorporates with His102 of the bovine $GABA_A$ receptor α-subunit(s), numbering based on α_1 sequence, (2) the major site of photoaffinity labelling by the partial inverse agonist, Ro15-4513, occurs on the $GABA_A$ receptor α-subunit(s), (3) the site of Ro15-4513 photoincorporation into the α-subunit(s) of the agonist-sensitive population of $GABA_A$ receptors occurs within amino acid residues 103 and the carboxyl terminus, distinct from the site for flunitrazepam photoincorporation, (4) the photoaffinity labelling site for Ro15-4513 on the agonist-insensitive cerebellar $GABA_A$ receptors (prior to residue Asn101, based on α_6 sequence) is unique compared to its site on the agonist-sensitive receptor population, (5) an intact disulfide bond and a free sulfhydryl group may be important for stabilizing the conformation of the binding site for the partial inverse agonist, and (6) the binding of the classical agonist is relatively insensitive to disulfide

reduction, but displays some sensitivity to alkylation of free sulfhydryl groups.

FUTURE DIRECTIONS

Careful scrutiny of the information gained from multidisciplinary studies will be necessary for a comprehensive knowledge, at the molecular level, of the mechanisms of action of GABA on $GABA_A$ receptors and of the actions of the allosteric modulators of $GABA_A$ receptor function. While some of the scientific endeavours that are required for the continued progress in the study of ligand-receptor interactions can utilize methodological approaches that are currently available, others will require advances in technical capabilities.

For example, the ability to visualize $GABA_A$ receptor proteins with the three-dimensional clarity available for soluble proteins by x-ray crystallography and NMR techniques is eagerly anticipated. Structural characterization with such high resolution will give the opportunity to understand, firstly, the structure of the protein and, secondly, the complex changes in the tertiary and quaternary protein structures that result from ligand interactions. Such conformational changes that are induced by the interaction of benzodiazepines and other ligands with the $GABA_A$ receptor could be resolved by comparisons of the "normal" and "modulated" states of the protein. A major goal is to identify the complete repertoire of amino acid residues that comprise the ligand binding domains and the determinants that are responsible for transducing allosteric signals through the complex multisubunit protein. To date, detailed structural analysis of

$GABA_A$ receptor proteins has been circumscribed by technical limitations, by the inability to crystallize these integral membrane proteins and by the very low abundance of the protein in brain tissue.

Another important area will be the development of technical strategies to characterize the subunit complement of $GABA_A$ receptors in various areas of the central nervous system and to establish the unique physiological and pharmacological properties of these receptor subtypes. With concerted efforts, the properties of native $GABA_A$ receptors from intact brain should be compared to the vast information available for recombinantly expressed receptors. The targetting of receptor subtypes with designer drugs, as referred to above, may be a realistic possibility if the specific functional attributes of receptor subtypes found in localized brain regions are elucidated.

In addition, kinetic analysis of ligand-receptor associations under physiological conditions is an investigative approach that may further elucidate the molecular mechanisms which underlie function. For example, the distinct mechanistic traits of benzodiazepine ligands with different efficacies may be identified by kinetic analysis of their interactions with $GABA_A$ receptors. In particular, such studies may substantiate the existence of multiple receptor protein conformations that are induced by the differential interactions of benzodiazepines with the $GABA_A$ receptor, thereby resulting in modulated channel function. Also, different states of the $GABA_A$ receptor-chloride channel complex may dramatically affect the action of benzodiazepines. Kinetic measurements of ligand binding and electrophysiological approaches capable of characterizing multiple receptor states may provide an avenue for such investigations. Although some of these directives

are currently possible, a vigorous kinetic study of benzodiazepine actions on the $GABA_A$ receptor has yet to be pursued.

In conclusion, the $GABA_A$ receptor is a complex multisubunit protein, and this complexity is compounded by the presence of multiple variants of each subunit class and the potential for post-translational modifications (Dunn et al., 1994; Macdonald and Olsen, 1994; Sieghart, 1995). It may be predicted, therefore, that the structural requirements underlying the allosteric mechanisms through which the benzodiazepine ligands interact with the receptor will be equally complex. Future investigations which give further information on these structural determinants will inevitably lead to a more comprehensive understanding of benzodiazepine modulation of $GABA_A$ receptor function.

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